Biodegradation

of Sodium Monofluoroacetate

(Compound 1080)

A thesis

submitted in partial fulfilment of

the requirements for the degree

of

Doctor of Philosophy in Microbiology

in

University of Canterbury

by

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University of Canterbury (1979)

Christchurch, New Zealand

CONTENTS

Publications:

1. Bong Chui Lien, Walker, J.R.L., Cole A.L.J., & Peters, J.A. (1979) Effect of sodium monofluoroacetate (Compound 1080) on the soil microflora. Soil Biol. Biochem. 11 : 13-18.

2. Bong Chui Lien, Walker, J.R.L. & Peters, J.A. (1980) The effect of fluoroacetate (Compound 1080) and fluoride upon duckweeds. N.Z.J.Sci. In press.

ACKNOWLEDGEMENTS

I would like to thank Dr J.R.L. Walker, Dr A.L.J. Cole and Dr J.A. Peters (Forest Research Institute, Rangiora, New Zealand) whose guidance in the microbiological, biochemical and ecological aspects of this project is very much appreciated. I am grateful to Dr Walker and Dr Cole for their helpful criticisms of the thesis, their patience extended during the preparation and their ready availability for consultation, and their tremendous support and encouragement throughout. Support and encouragement has also come from my fellow post-graduate colleagues (Room 233).

I would also like to thank Mrs A. Luney and Mrs M. Stevens who have made the laboratories pleasant places to work in. Thanks also to Mrs H. Langer and Miss Fiona Lees for their assistance in statistical analysis of some of the results presented.

I also wish to express my gratitude to New Zealand for giving me the opportunity to study here : in particular, the following organisations which provided financial assistance - The Miss E.L. Hellaby Indigenous Grasslands Research Trust for a scholarship (1978) and the Agricultural Pest Destruction Council for research grants and scholarships; and also to Mr A.I.R Jamieson and family (Christchurch) for their having given me 'a home away from home'. I am most indebted to my mother and family.

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ABSTRACT

The biological potential of some South Island soils for defluorination of sodium monofluoroacetate (NaFa) was examined. In these soils were found a diversity of bacteria: *Pseudomonas* sp., *Bacillus subtilis*, *Aeromonas* sp., *Actinobacillus* sp., *Citrobacter* sp., *Staphylococcus aureus* and *Lactobacillus* sp. Also found were the fungi: *Acremonium strictum*, *Fusarium solani*, and *F. oxysporum* which showed the ability to cleave the carbon-fluoride bond of fluoroacetate. NaFa-tolerant organisms were also isolated from these soils and include *Chlorella* sp., and the fungi: *Penicillium* sp., *Aspergillus* sp., and *Mucor* sp. Of these organisms, the fungi were selected for more detailed study of the influence of environmental factors on their growth and defluorinating activity. Factors which favoured growth also favoured the defluorination of NaFa. Growth of *F. solani* and its defluorination of NaFa increased with increasing temperature from 10°C to 30°C. While growth of *F. solani* showed an optimal pH plateau from 5.8 - 7.2, defluorination of NaFa was more sensitive to external pH with a sharp optimum at 5.8. Studies on the effect of nitrogen sources (NO_3^-, NH_4^+, and urea) showed that both the growth and defluorination of NaFa were maximal in NH_4^+-N medium up to 448 mg N l^{-1} whereas such effect on *A. strictum* was not evident. Further increase in NH_4^+-N concentration was inhibitory to both growth and NaFa defluorination. The availability of additional carbon sources (acetate, glucose) enhanced growth and NaFa defluorination. The degree of enhancement of defluorinating activity decreased with increasing concentration of the supplementary carbon source in NaFa-medium for F. *solani*, whereas the response of *Penicillium* sp., in terms of growth and NaFa defluorination increased with increasing glucose concentration. The ability of fungi to degrade NaFa was induced. From assays of the culture filtrate, and various cell fractions, it was shown that defluorination of NaFa took place intracellularly. The regulation of growth and defluorination of NaFa by environmental factors is discussed.

Studies of NaFa defluorination were pursued at cellular and enzymatic levels. The defluorinating enzyme, haloacetate

halidohydrolase or fluorohydrolase, extracted from *F. solani* and *Pseudomonas* sp. were similar in their molecular weight, estimated to be between 45,000 - 68,000. Both enzymes catalysed the breakdown of NaFa to F^- and glycolate on a mole to mole basis. The fluorohydrolase was highly specific to monohalogenated acetates, showing a higher specifity for fluoroacetate and fluoroacetamide. Fluorohydrolase activity was inhibited by thiol-alkylating agents suggesting the involvement of -SH groups at the active site of the enzyme. The mechanism of enzymatic defluorination of NaFa was discussed. The Q_{10} values and the response to pH of NaFa defluorination by cells and enzyme were different and their difference was discussed in terms of ionisation of fluoroacetate and their rate of diffusion into the cells.

Phytotoxicity studies with *Chlorella* sp., and three species of duckweeds: *Spirodela oligorrhiza, S. polyrrhiza* and *Lemna minor* showed that the alga was highly tolerant while the duckweeds were extremely sensitive to the presence of NaFa. Whilst growth of *Chlorella* sp. was unaffected in 20mM NaFa medium, growth of the duckweeds was completely suppressed in 0.5-1.0 mM NaFa medium. The tolerance or susceptibility of plants to NaFa is discussed.

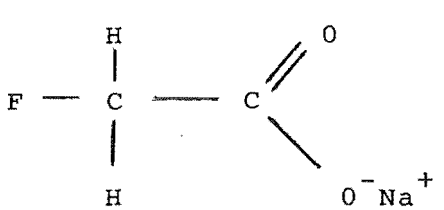
CHAPTER ONE

SODIUM MONOFLUOROACETATE (NaFa)

1.1 INTRODUCTION

Sodium monofluoroacetate[1], commonly known as "Compound 1080" (henceforth abbreviated as NaFa), is a fluorine-substituted acetate first synthesised by Swartz in 1896 (Pattison, 1959). It is used world-wide as a general mammalian pesticide.

Sodium monofluoroacetate



1.2 HISTORICAL BACKGROUND AND DEVELOPMENT OF SODIUM MONOFLUOROACETATE AS A PESTICIDE

An interesting and detailed account of the historical background of NaFa has been given by Pattison (1959). Toxicity of NaFa was not mentioned when the synthesis of the compound was first published. However it was patented as a moth-proofing agent in 1930 in Germany. The toxic nature of fluoroacetates was first discovered independently by Schrader of Germany, and a group of Polish chemists led by Grszkiewicz-Trochimowski during the 1935-1939 period. Their work showed that fluoroacetates were extremely poisonous to warm-blooded animals and this discovery had serious implications in the era prior to World War II. Consequently the Polish chemists systematically studied toxicities of fluoro-organic compounds and came to the conclusion that FCH_2COO^- was the toxic radical.

When World War II broke out, Sporzynsky, one of the Polish chemists, escaped to England and turned over their results to the British. A team of scientists led by McCombe and Saunders (Pattison, 1959; Saunders, 1972) were then actively engaged in research on chemical warfare agents. Extensive research on fluoro-organic compounds was subsequently initiated in Britain. They came to the same conclusion as the Polish chemists; i.e., that any fluoro-organic compounds which could give rise to FCH_2COO^- would be toxic. This led them to the generalisation that any straight chain fluoro-organic compounds with even number of carbon atoms would be toxic (via hydrolysis or β- oxidation) whilst those with odd numbers were relatively non-toxic. Thus a distinct relationship exists between chemical constitution and physiological action of fluoro-organic compounds.

During World War II, research results were exchanged between Great Britain and United States of America. America was then stricken with a chronic shortage of rodenticides (thallium, red squill and strychnine) (Atzert, 1971; Pattison, 1959). The demand for rodent control was particularly urgent to combat rodent-borne diseases such as bubonic plague and typhus fever and to reduce economic losses. NaFa was among the ten chemicals tested and evaluated as potential rodenticides (Ward, 1946;

Atzert, 1971). Laboratory tests showed that NaFa was less toxic to man than either strychnine or arsenous acid, but more toxic than thallium sulphate, zinc sulphide and barium carbonate. Field trials on the efficiency of NaFa as a rodenticide and general mammalian pesticide were carried out in Denver (Robinson, 1949)and other parts of America (Emlen & Stokes, 1947), in England (Peacock, 1964), in Australia (Meldrum *et al*, 1957), in Peru to control bubonic plague (Macchiavello, 1946), and in New Zealand (Staples, 1968). A high efficiency of killing was recorded in all trials.

1.3 USAGE OF NaFa

Considerable losses in food and other agricultural products are caused in part by animal pests: in North America (USA, Canada, Mexico), predation of cattle and sheep, and depredation of fodder land by coyotes (Robinson, 1949; Peacock, 1964); by rabbits in Australia and New Zealand (Meldrum *et al.*, 1957; Stables, 1968); and damage by rodent infestation of crop orchards and grain storage (Hilton *et al.*, 1969). Deterioration of exotic forests and soil erosion by animal grazing, gnawing and burrowing are also serious ecological concerns. Forest regeneration is severely hampered by heavy grazing on trees by opossums and rabbits (*Trichosorus vulpecula* and *Oryctolagus cuniculus*) in New Zealand (APDC Report, 1979; Rammell & Fleming, 1977) and destruction of conifer seeds by rodents (McKeever, 1962). The burrowing activity of rabbits also aggravates the problem of soil erosion.

Nowadays NaFa is used as a general mammalian pesticide by qualified operators to control animal pests. It is used less frequently as insecticide on non-edible cash crops in England (David & Gardiner). In Australia and New Zealand the animal pests being controlled are chiefly rabbits and opossums, secondarily such pests as wallabies. These pests exert considerable pressure on grazing: approximately 8 rabbits, or 10 opossums, or one wallaby graze as much as one sheep. Another risk posed by opossums is in transmission of bovine tuberculosis (Batcheler, 1978). NaFa has been used in increasing quantity in New Zealand since 1957 amounting to 2.86 tonnes/year in 1976 (Batcheler, 1978).

1.4 PHYSICAL AND CHEMICAL PROPERTIES OF NaFa

There are several features of NaFa which make it a superior mammalian pesticide. NaFa is colourless and odourless which makes it undetectable. Ready solubility in water renders it easy to use. It is quick in killing action (within ½ to 1 hour of consumption).

Chemically fluoroacetates have several properties which set them apart from other halogen-substituted acetates. Fluoroacetate is the most stable and most toxic of all halogenated aliphatic acids. The carbon-fluorine bond is shorter and stronger than other carbon-halide bonds as indicated by the internuclear distance, dissociation constants and bond energy as shown in Table 1.1.

Table 1.1: Properties of Halgenated Aliphatic Acids.

Adapted from Pattison (1959)

Aliphatic acids XCH_2COOH	Atomic Radii A^o (nm)	Internuclear distance X-C, A^o (nm)	Bond Energy kcal/mole (kJ/mole)	Dissociation Constant Ka x 10^{-5}	Electroneg-activity of X
HCH_2COOH	.29	1.14	87.3	1.8	
	(0.029)	(0.114)	(365.4)		
FCH_2COOH	0.64	1.45	107	217	4.0
	(0.064)	(0.145)	(447.9)		
$ClCH_2COOH$	0.99	1.74	66.5	155	3.0
	(0.099)	(0.174)	(278.4)		
$BrCH_2COOH$	1.14	1.90	54.0	138	2.8
	(0.114)	(0.190)	(226.0)		
ICH_2COOH	1.33	2.12	45.5	75	2.5
	(0.133)	(0.212)	(188.4)		

The oral toxicities (LD_{50}) of halogenated aliphatic acids to white mice were studied by Morrison (1946) and were found to decrease in the order :

$$ICH_2COOH > BrCH_2COOH > ClCH_2COOH$$

The toxicities bear an inverse relationship to the electronegativities of these compounds. Such compounds exert their effect as thiol acetylating agents. Fluoroacetate is an exception to the rule. The stability of C-F bond makes it impossible to thiol acetylate the cysteine of enzymes. The mechanism with which fluoroacetate exerts its toxic action is associated with the atomic radii of fluorine and hydrogen atoms, which are sufficiently similar to confer a remarkable resemblance in the spatial configuration of CH_3- and CH_2F- radicals. Consequently fluoroacetate can mimic acetate in its conversion to acetyl CoA (or fluoroacetyl CoA) which subsequently enters the tricarboxylic acid cycle to be synthesised into a lethal compound, fluorocitrate (Peters, 1957).

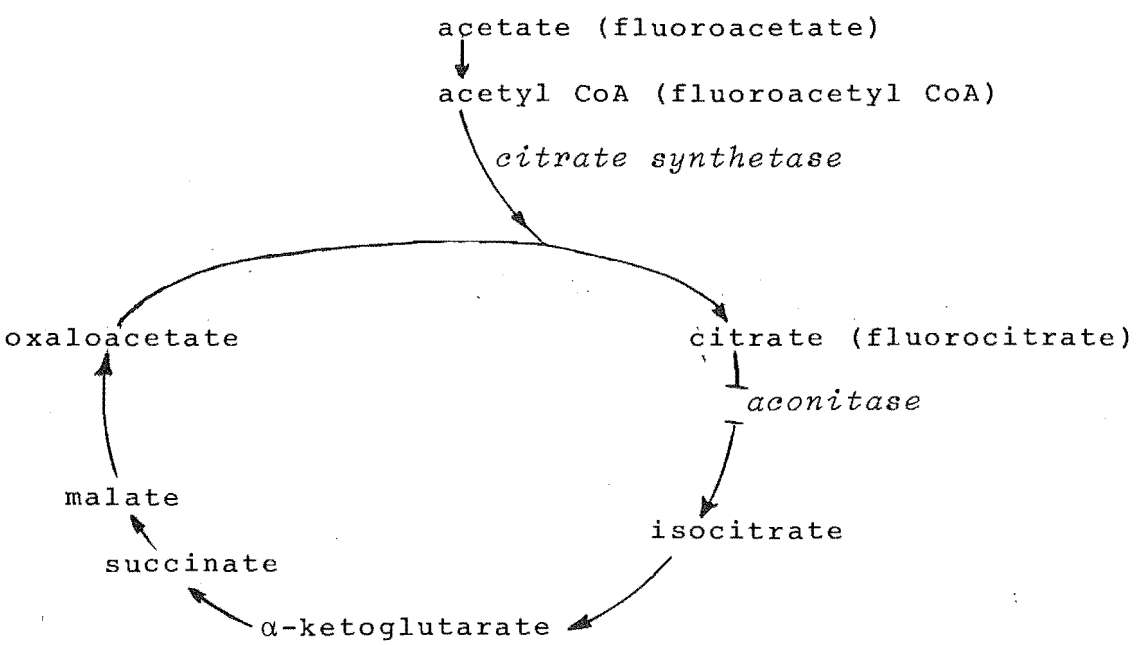
1.5 MODE OF ACTION NaFa

FCH_2COO^- was identified as the toxic radical in the early 1940s. But the mode of action of fluoroacetate remained a subject of great interest, because unlike the other halogenated acetates, fluoroacetate was found to have no inhibitory effect on isolated enzymes (Bartlett & Barron, 1947; Liebecq & Peters, 1949) Working with sliced liver tissues Bartlett & Barron (1947) found that fluoroacetate caused acetate accumulation. They advanced the theory that fluoroacetate acted as a competitor of acetate, thus blocking the conversion of acetate to acetyl CoA (then known as "activated acetate"). The theory was supported by evidence based on inhibition of respiration of yeasts and bacteria in the presence of fluoroacetate. Kalnitsky & Barron (1947) showed that the inhibition of O_2-uptake by yeasts could be reversed by supplying a higher concentration of acetate. A year later, the same authors observed marked accumulation of citrate in rabbit kidney homogenate treated with fluoroacetate.

Inhibition of O_2-uptake by tissues, citrate accumulation in the presence of fluoroacetate, and the lack of inhibitory effect on isolated enzymes were observations confirmed by Liebecq and Peters (1949), working with guinea pig kidney homogenates. They also showed that fluoroacetate could block

fumarate oxidation without acetate accumulation, and that the inhibition was proportional to fluoroacetate applied; this was also accompanied by increasing accumulations of citrate. They further examined the effect of fluoroacetate on each isolated reaction of the tricarboxylic acid (TCA) cycle. None of the reactions were inhibited when studied singly and yet the cycle was blocked somewhere below the citrate level. They suggested that fluoroacetate itself was not an inhibitor *per se*, but that "lethal synthesis" had occurred during which fluoroacetate was transformed into fluoroacetyl CoA, condenses with oxaloacetate to form fluorocitrate which competes with citrate for the active site on the enzyme, aconitase, thus blocking the conversion of citrate to isocitrate (Fig 1.1.)

Figure 1.1: Mode of Action of NaFa



Fluorocitrate also competitively inhibits succinate dehydrogenase (Fanshier *et al.*, 1964). The resulting high concentration of citrate also blocks glucose metabolism via allosteric inhibition of phosphofructokinase (Elliott & Phillips, 1954; Dunn & Berman, 1968). Consequently energy supply is gradually diminished to a point where permeability barriers are destroyed and cellular functions impaired. In animals this is manifested as nervous convulsions and cardiac arrest. The organism eventually dies from energy depletion.

1.6 TOXICITY OF NaFa

The first comprehensive review of toxicity of NaFa was made by Chenoweth (1949). NaFa is highly poisonous to warm-blooded animals, but considerably less so for cold-blooded animals (Table 1.2). The LD_{50} (probability that 50% of the population would be killed) for the African clawed toad is more than 8,000 times that for the dog on a weight to weight basis. The pharmacological actions are equally varied with deaths resulting from one or more of the following:

1. Respiratory arrests following severe convulsions - herbivores are mainly affected this way;
2. Gradual cardiac failure; and
3. Progressive depression of central nervous system followed by cardiac and respiratory failures - typical reaction of carnivores.

Death of omnivores tend to result from disorders of both the heart and central nervous system.

From limited data published, fishes were shown to be remarkably tolerant to NaFa. Fingerling trout survived when force-fed with 4-8 mg NaFa (Rammell & Fleming, 1977).

Insects have been shown to be susceptible to NaFa. Secondary poisoning of fleas fed on NaFa-poisoned rats was observed by Macchiavello (1946). From studies of the potential of NaFa as an insecticide, David & Gardiner (1951, 1953, 1954). reported that *Aphis fabae*, larvae of *Pieris brassicae* and *Phaedon cochlearie* were killed from infesting plants grown in soil treated with NaFa. Ants, roaches and larvae of *Anopheles* are also sensitive to NaFa (Chenoweth, 1949). Bees are also highly susceptible as reported by McIntosh *et al.*, (1964) that 18,000 bees were killed by 9.45g of NaFa.

Phytotoxicity of NaFa was studied in relation to its use as a systemic insecticide. The responses of plants to NaFa treatment vary from leaf necrosis, stunted growth and wilting (David and Gardiner, 1951, 1953, 1954, 1955; Peters, 1957; Cheng *et al.*, 1968; Cooke 1976c). However, there are reports that some plants are able to split C-F bond of fluoroacetate

Table 1.2: Toxicity of Sodium Monofluoroacetate

	LD_{50} mg kg^{-1} body weight	Route of administration	References
Man	2.0-5.0	oral	Chenoweth, 1949
Monkeys	4.0-15.0	oral	Chenoweth, 1949
Herbivores			
Cattle	0.2-0.4	oral	Robinson, 1970
Sheep	0.2-0.5	oral	Jensen *et al.*, 1948
Dogs	0.06	intravenous	Chenoweth, 1949
	0.1-0.1		Ward, 1946
Cats	0.2	intravenous	Chenoweth, 1949
	0.3	intraperitoneal	Ward, 1946
Coyote	0.1	intravenous	Chenoweth, 1949
	0.2	intraperitoneal	Ward, 1946
Rodents			
Norway wild rats	3.0	oral	Chenoweth, 1949
House mouse	8.0	oral	Chenoweth, 1949
Field mouse	4.0	oral	Ward, 1946
Rabbits (New Zealand white)	0.25	intravenous	Chenoweth, 1949
Oryctolagus cuniculus	1.2-1.6	oral	Peters, 1970
Marsupials: Opossums			
Didelphis marsupiates	60.0	oral	Atzert, 1971
Trichosorus valpecula	1.2-1.6	oral	Peters, 1970
Australian opossums	100.0	oral	Batcheler, 1978
Birds: Magpie	0.6-1.3	oral	Ward & Spencer, 1947
Chicken	7.5	oral	Chenoweth, 1949
Frogs: *Rana pipiens*	150.0	subcutaneous	Chenoweth, 1949
South African bull frog (*Xenopis laevis*)	500.0	intraperitoneal	Chenoweth, 1949

(Preuss *et al.*, 1968; Preuss & Weinstein, 1969; Ward, 1973), and this will be reviewed in Chapter 5.

Data on toxicity of NaFa to micro-organisms come chiefly from studies made to elucidate the mechanisms of biochemical reactions, and investigation of the potential use of NaFa as a chemotherapeutic agent. The latter was aimed at controlling viruses. Viruses have been shown to be resistant to NaFa treatments (Pattison, 1959). The metabolism of acetate by yeasts (Kalnitsky & Barron, 1947; Aldous, 1963) was inhibited by 95% in the presence of 1.0 mM NaFa whilst 2.0 mM NaFa completely inhibited pyruvate and acetate metabolism by such bacteria as *Corynebacterium creatinovorans* and *Escherichia coli* (Black & Hutchens, 1948), and *Pseudomonas* ODI (Jayasuriya, 1956). Dagley and Walker (1956) reported that susceptibility of *Vibrio* 01 to NaFa was influenced by the substrates to which the vibrio cells were adapted. Sensitivity, as influenced by carbon substrates on which micro-roganisms are grown, has also been corroborated by Cox and Zatman (1976). They reported that the growth of a facultative methylotroph is ten times more sensitive to NaFa inhibition when grown on non-C_1 compounds. Effect of NaFa on fungal growth was reported to be inhibitory to *Aspergillus terreus* (Peters, 1957) and *Physarella oblonga* (Chenoweth, 1949). Some species of *Pseudomonas* have been reported to degrade the compound and use it as the carbon source for growth (Kelly, 1965; Tonomura *et al.*, 1965; Goldman, 1965)

1.7 OCCURRENCE OF MONOFLUOROACETATE IN NATURE

Fluroacetate was considered a synthetic chemical until 1944 when Marais reported that it occurred naturally in South African woody shurb, *Dichapetalum cymosum*, locally known as "Gifblaar" (Marais, 1944). The discovery was a culmination of years of research into the toxicity of "Gibflaar" and other poisonous plants of South Africa which caused considerable stock losses. Since then occurrence of fluoroacetates in other poisonous plants had been widely reported: in the seeds of *D. toxicarium* (Peters *et al.*, 1960) in Siera Leone, *Acacia georginae* (Murray *et al.*, 1961; Oelrichs & McEwan 1961, 1962), in other leguminous plants - *Gastrolobium grandiflorum* (McEwan,

1964) and *Oxylobium parviflorum* (cited by Alpin, 1971) in Australia; and *Palicourea marcgravii* St. Hil in Brazil (De Oliveira, 1963). In New Zealand, the toxicity of *Galega officinalis*, which belong to the same family (Papilionacae) as *Gastrolobium* and *Oxylobium*, established on the banks of Manawhatu River is reportedly due to fluoroacetate (Peters, 1970).

Where fluoroacetate has been identified in plants as the toxic compound, the content may amount to 0.0125% on a dry weight basis. Thus 2 g of fresh leaves is sufficiently toxic to kill an adult sheep (Aplin, 1971). In Australia records of stock losses due to accidental grazing on these poinsnous plants dates as far back as 1837. 4,000 head of sheep and 100 head of cattle may be killed by grazing on *O. parviflorum* in a year. Cases of secondary poisoning of dogs and cats from consuming entrails of poisoned marsupials and birds are still common.

The concentration of fluoroacetate in plants varies widely from species to species, within the species and seasonally within individual plants. Generally fluoroacetate content is highest during active growth period (Badenhuizen, & Slinger, 1954; Vickery & Vickery, 1972; Aplin, 1971). The concentration varied from 10-25 mg fluoroacetate kg^{-1} to 120 mg kg^{-1} dry plant tissue in *A. georginae* (Murray *et al.*, 1961; Oelrichs & McEwan, 1962) and *G. grandiflorum* respectively (McEwan, 1964) to 789 mg kg^{-1} in *D. cymosum* (Marais, 1944). These values were calculated on the assumption that all fluoride present was in the form of sodium monofluoroacetate.

Perhaps of less economic impact, but of some environmental concern is the finding that plants which do not normally synthesise fluoro-organic compounds could be induced to do so when exposed to environment high in inorganic fluoride content. Monofluoroacetate and fluorocitrate had been detected in crested wheat grass growing within the vicinity of a phosphate plant which emits fluoride (Yu & Miller, 1970). Traces of these compounds were also found in a commercial specimen of oatmeal (Peters & Shorthouse, 1972a).

1.8 CURRENT RESEARCH ON NaFa

Controversy still surrounds the use of NaFa as a pesticide despite almost 35 years of extensive world-wide application. Objections to its use stem primarily from economic concern and considerations of the environmental and ecological impact. NaFa is a highly unselective poison and cases of accidental poisoning of sheep and cattle (Robinson, 1970; Batcheler, 1978) and desirable native fauna are occasionally reported. Furthermore, there is no antidote for NaFa-poisoning. Consequently researchers into the above problems are directed towards developing an antidote and a method of applying the pesticide in such a way as to minimise risks to non-target animals. Therefore research on the use of NaFa may be categorised thus:

1. Search for an antidote;
2. Ecological impact on native fauna;
3. Formulation of poison baits;
4. Persistence of NaFa;
5. Phytotoxicity of the compound; and
6. Improved techniques on assaying or analysis of NaFa.

The search for an antidote was begun in early 1940s (Peters, 1954) when it was imperative in case NaFa was used as chemical warfare agent during World War II. Interest in this aspect of research is maintained to present day because of occasional huge stock losses due to NaFa-poisoning either from grazing on poisonous shrubs or from poisoned baits. No effective antidote has yet been found. Current treatment is to infuse the victims with monoacetin and glucose after being administered with anticonvulsants. The treatment is generally symptomatic. Since the use of NaFa as a pesticide, there have been 16 reported cases of human fatalities on consumption of NaFa in USA (Atzert, 1971), 2 non-fatal cases and one confirmed fatality due to NaFa-poisoning in New Zealand (Batcheler, 1978).

The ecological impact on native fauna was investigated when NaFa was first used in trials against coyotes in Denver, Colorado (Robinson, 1949). Similar investigations have been carried out in Australia and New Zealand. These investigations include identification of susceptible species and their

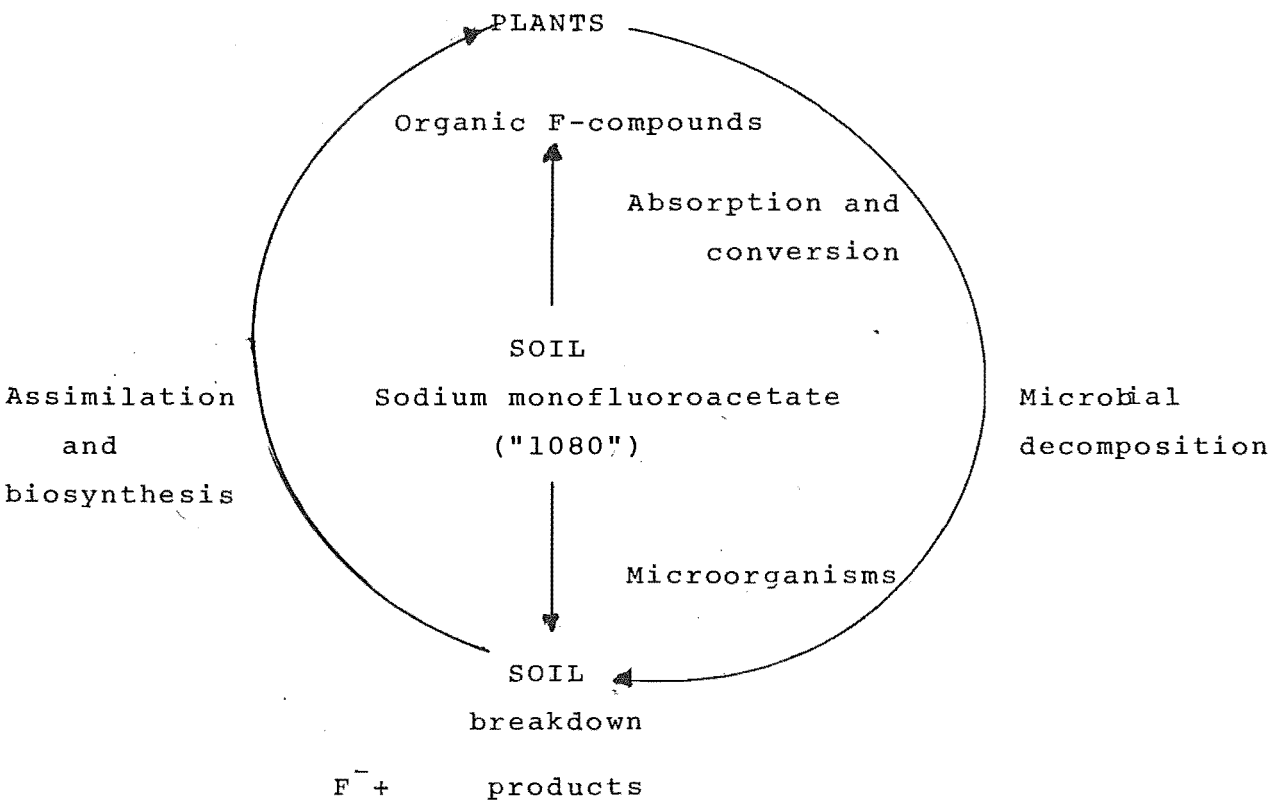
ecological behaviours. Understanding of animal and bird behaviours could help the timing of application of NaFa to minimise risks to non-target species and maximise efficiency against pests. Increased efficiency depends also on the kinds of poisoned baits used and the target pests. In America where coyotes are the main target, NaFa is incorporated into meat; and in Australia and New Zealand, the usual baits are carrots, pollards, brans and molasses previously soaked in NaFa solution. Use of Nigrosine or lissamine green dye to mask the appearance of baits to increase bird aversion to the baits is one of the methods used to reduce accidental poisoning of birds (Meldrum *et.al.*, 1957; Batcheler, 1978).

While extensive research on the above problems is being conducted, little is known on the persistence of NaFa in soils or in waterways. However soil pseudomonads which were able to degrade NaFa had been isolated (Kelly, 1965; Goldman, 1965; Tonomura *et al.*, 1965). It was demonstrated that NaFa treated soils lost their toxicity within 11 weeks (David & Gardiner, 1966). In comparison physical weathering of poison baits has received greater attention in the recent past than biodeterioration of NaFa (Staples, 1968; Corr & Martire, 1971).

One of the reasons for limited studies on persistence of NaFa and screening of poisonous plants containing NaFa could lie in the lack of a rapid method for detecting NaFa for routine analysis. Identification and quantification of fluoroacetate were deduced from chemical tests and fluoride analysis (Marais, 1944). Biological material has to be combusted, distilled in acid, inorganic fluoride separated from organic fluoride and the latter is either chromatographed on paper chromatography, or gas-liquid chromatography against pure standards of fluoroacetate. (Yu & Miller, 1970; Lovelace, *et al.*, 1968; Ward,1973; Hall & Cain, 1972).

The fate of NaFa in the environment can be summarised as in Figure 1.2. This project will be concerned with investigations of:

Figure 1.2: Fate of NaFa



1. Microbiological potential of some New Zealand soils in degradation of NaFa;
2. Effect of environmental factors on NaFa-biodegradation
3. Biochemical aspects of NaFa-biodegradation; and
4. Phytotoxicity studies with duckweeds.

Knowledge gained from the above studies may contribute towards a safer and more efficient method of application.

CHAPTER TWO

BIOLOGICAL POTENTIAL OF SOILS FOR NAFA DECOMPOSITION

CHAPTER TWO

BIOLOGICAL POTENTIAL OF SOILS FOR NAFA DECOMPOSITION

2.1 INTRODUCTION

The toxicity of NaFa lies in the intact C-F bond. The decomposition of NaFa was followed by measuring inorganic fluoride ions (F^-) released into the soil or culture medium. Two methods of quantitative analysis of free F^- were tested and compared. The biological breakdown of NaFa was determined by isolation of NaFa-degrading micro-organisms and enumeration of microbial populations using standard microbiological techniques. The effect of F^-, breakdown product of NaFa on soil microbial defluorination of NaFa was also studied.

2.2 MATERIALS AND METHODS

2.2.1. Quantitative Analysis of Fluoride Ions

a. Colorimetric method - The method used was developed by Bellack and Schouboe (1958), and utilises the principle of decolorisation of a red-coloured compound by fluoride ions. The coloured complex was produced from reaction of zirconyl ions($ZrO^{=}$) and 2-(p-sulphophenylazo) - 1,8-dihydroxynaphthalene -3,6- disulfonate (SPADNS). The colour was read on an EEL colorimeter using a 540 nm filter. A calibration curve was prepared using standard NaF solutions, made up in distilled water(0.0-2.0 mg $F^-.l^{-1}$) or in basal medium (0.0-1.4 mg $F^-\ l^{-1}$).

b. Potentiometric Method - This method was developed by Orion Research Incorporated (1973), based on ionic activity or potential generated by free F^-. This potential is measured by a combined F^--specific electrode (Orion model 96-09) and registered on an Orion model 407 specific ion meter. The F^--specific electrode contains a lanthanum fluoride crystal membrane in which only fluoride ions are mobile; thus the electrode responds only to free unbound or uncomplexed F^-. The F^--specific electrode was calibrated with known NaF standards made up in phosphate buffer (20 mM) at pH 6.8.

2.2.2. Soils

The soils used in present study were all "gley, stony silt loam and sand" except one sample which was a humus layer (personal communication, J.A. Peters, Christchurch, N.Z.). They were sampled from six districts in the South Island (Figure 2.1), mostly around farm airstrips where NaFa baits were loaded into aircraft for aerial application. These sites had therefore been previously exposed to high localized amounts of NaFa contamination. The Oweka site was selected for a more extended study of the effect of NaFa contamination upon surrounding soils. Soils sampled from Oweka differed in their previous exposure to NaFa contamination: from NaFa bait-loading and preparation areas, relatively uncontaminated area at a distance from bait preparation and loading sites, and from a nearby farm in which NaFa had never been used but which previously received high superphosphate fertilizer treatment.

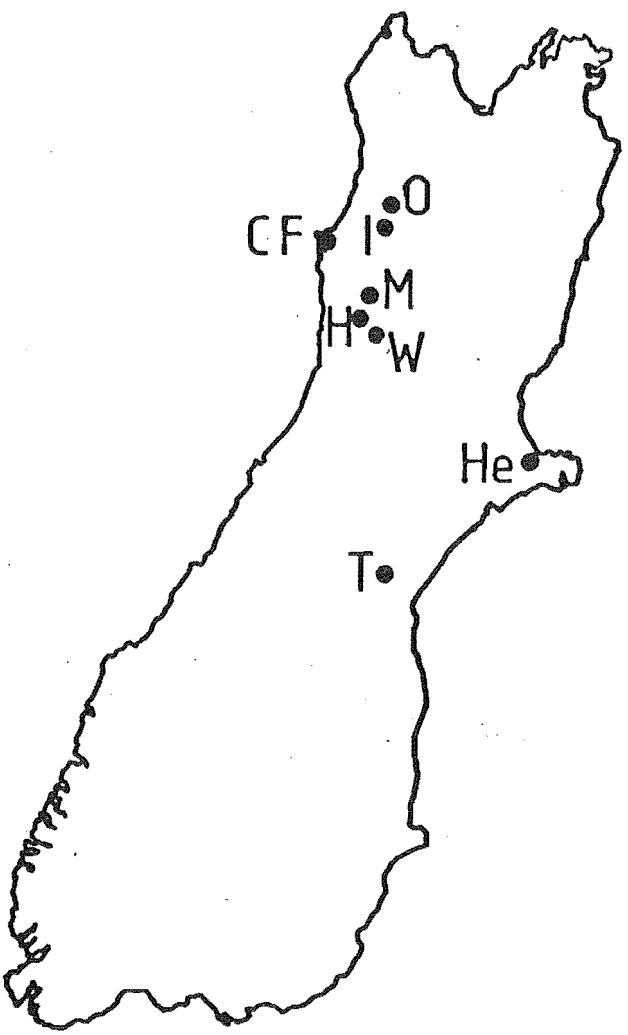
For a study of persistence of NaFa in the above soils, freshly collected soils were used whilst for a study on the relationship between microbial proliferation and NaFa decomposition finely sieved air-dried soils were used. NaFa-degrading micro-organisms were also isolated from finely ground air-dried soils.

2.2.3. Enrichment and Culture Media

NaFa-degrading micro-organisms were selected by enrichment culture on a mineral salt medium containing 20mM NaFa, 2.0 KH_2p04, 1.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$ per litre of distilled water. For isolating soil fungi, the above medium was supplemented with 10.0 mg $CaCl_2$, 0.2 mg $FeSO_4$ per litre of distilled water. The pH of mineral salts medium was adjusted to 6.8 with NaOH for bacteria cultures or 5.6 for experiments with fungi. The medium was sterilised by autoclaving at 120°C for 20 mins. Sterilised $MgSO_4$ solution was added separately to the above medium so that the final concentration of $MgSO_4$ in the medium was 1.0 g l^{-1}.

Microbial colonies were plated out, isolated and maintained on 'BBL' nutrient agar (bacteria), 'Oxoid' potato-dextrose or potato-sucrose agar and 'Difco' malt-extract agar (fungi), and NaFa mineral salts medium solidified with

FIG 2.1 SOUTH ISLAND : DISTRICTS FROM WHICH SOILS WERE SAMPLED FOR STUDYING DEFLUORINATING POTENTIAL.



Keys :

CF : Cape Foulwind	M : Mawheraiti
H : Hukarere	O : Oweka
I : Inangahua	T : Taiko
He : Heathcote	W : Waipuna

1.5% Davis (N.Z.) agar. The basal medium described by Bollard (1966) was used for algal and duckweed cultures.

2.2.4a Persistence of NaFa in soils

Samples (250 g) of all freshly collected soils were mixed with 2.5 g NaFa suspended in 20 ml distilled water, by shaking. For controls, sterilised soils similarly treated with NaFa in 20 ml sterile distilled water were used. Soils were sterilised by autoclaving at 120°C for an hour for 3 successive days. Unsterilised NaFa-treated soils in plastic bags and sterilised soils in 1-litre flask were incubated together with unsterilised, untreated soils in plastic bags at 25°C for 98 days. At intervals a 25 g sample was withdrawn and shaken with 125 ml basal medium for 30 mins, and the concentration of F^- measured using F^--specific electrode. Soil particles were then removed by filtration through Whatman No 1 paper. The clear soil solution was divided in four 30-ml aliquots, dispensed into 250-ml conical flasks, two of which were autoclaved and the other two remained unsterilised. Soil solution prepared from sterilised soil was autoclaved after F^- concentration was measured. Soil solutions were returned for further incubation for 10 days, and F^- concentration was again measured.

b. Defluorinating acticity of soils - Triplicates of 10-ml aliquots of sterilised 20 mM NaFa mineral salts medium were inoculated with 0.2 g air-dried soil sample from 7 sites at Oweka. These were incubated in the light at 25°C on an orbital shaker, at 300 rev $min.^{-1}$ Defluorination of NaFa was followed by measuring F^- concentration in the medium over a period of 20 days, and 20 mM NaFa was added at intervals. Microbial growth was followed by sampling 0.1 ml from each culture flask, serially diluted, plated out on agar plates and incubated at 25°C for 10 days. Only dilution plates which gave total microbial counts of 30-300 were retained.

c. Effect of NaF on soil microbial defluorination of NaFa

A series of mineral salts media containing different NaF concentrations (0-100 mM) was prepared. Four replicates of 20-ml aliquots in plastic containers were inoculated with 0.2 g air-dried Oweka soil each. To two of the replicates, NaFa was added to make the final concentration up to 20 mM. These were

incubated at 25°C, without shaking for 12 days, then F^- concentration measured.

2.2.5. Isolation and Purification of NaFa-degrading Micro-organisms

For the isolation of NaFa-degrading microorganisms from soils, 10 ml aliquots of 20 mM NaFa mineral salts medium were inoculated with 0.2 g air-dried soil and incubated at 25°C for 20 days. Microorganisms were isolated by sampling 0.1 ml culture solution from each flask, serially diluted and plated out on solid culture media described in 2.2.3. Bacterial colonies from dilution plates were selected on the basis of general colony appearances: color, shape and texture of the colony, size and shape of the cells, and response to Gram-staining. A representative of each type was isolated, purified by repeated streaking and subculturing on nutrient agar plates. Fungal species were purified by streaking the spores produced on PDA plates. The plates were incubated at 25°C for 3-5 days or the shortest period until small colonies were visible and before the organism had spread all over the plates when the chance of cross-contamination was high. Repeated transfer and subculture on fresh agar plates was used to purify the isolates.

The bacteria isolated were kindly identified by Mr R.C. Bridger, (Godfrey Laboratory, Christchurch, New Zealand), and the identification of the fungal isolates was confirmed by Dr C. Booth and Dr B.L. Brady (Commonwealth Mycological Institute Surrey, England). The ability of each purified isolate to defluorinate NaFa and use it as the sole carbon source for growth was tested. A suspension of bacterial cells was prepared from each isolate. The concentration of the inoculum was adjusted to approximately 10^7 cells ml^{-1}. Triplicates of 10 ml aliquots of 20 mM NaFa mineral salts medium were inoculated with 0.1 ml of the cell suspension, and incubated at 25°C for 7 days without shaking; F^- released into the medium was then measured.

2.3 RESULTS

2.3.1. Comparison of Colorimetric and Potentiometric Methods Of Fluoride Ion Determinations

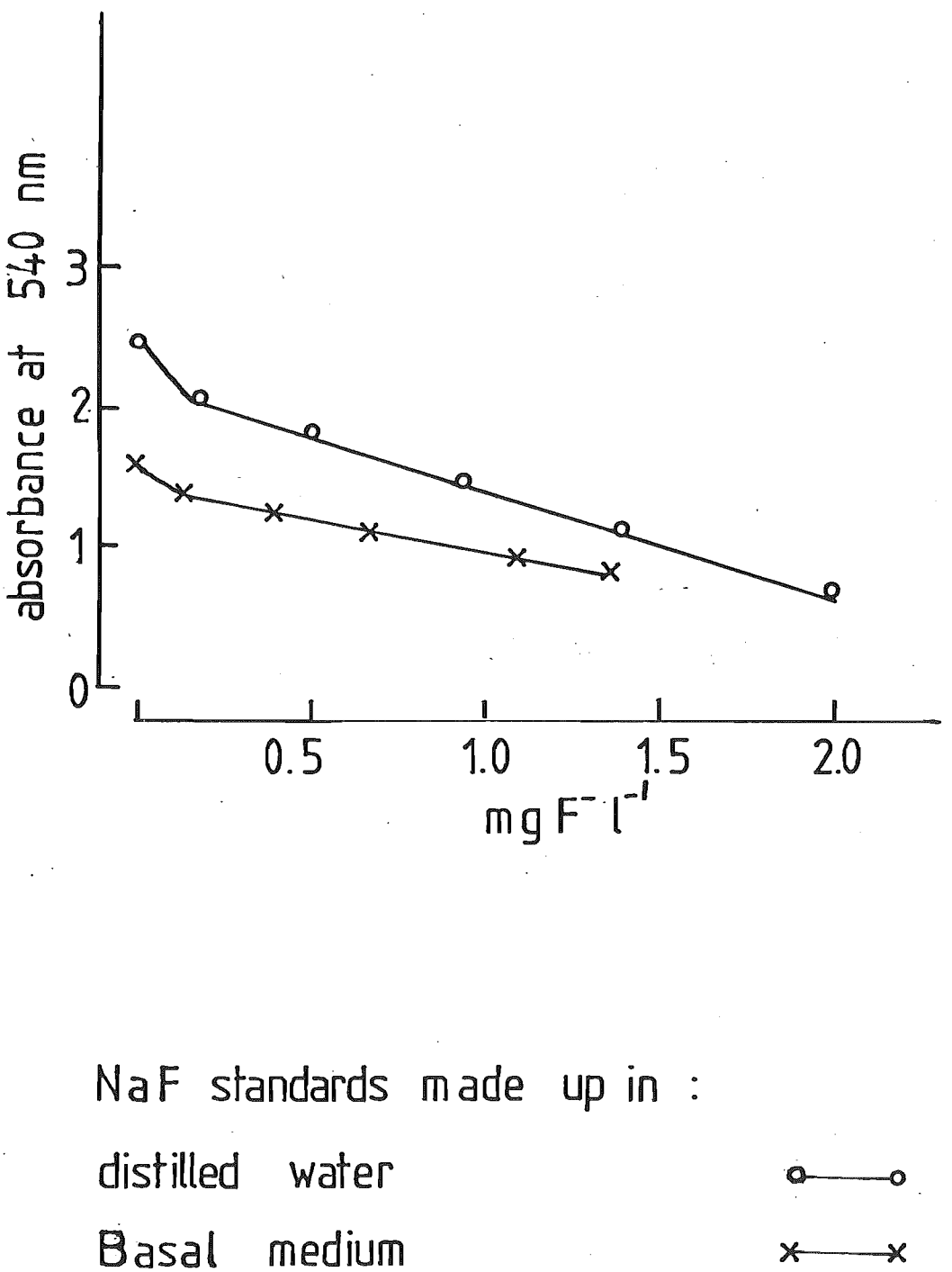
The relative reliability of colorimetric and potentiometric methods of fluoride ion determination was compared, using NaF solutions made up in distilled water and basal medium. As shown in Fig. 2.2, the colorimetric method was less sensitive to fluoride ions in basal medium as indicated by the more gentle slope. However, there was no major discrepancy in the fluoride ion reading as measured with the F^- -specific electrode except for NaF concentration which is smaller than 10 µM as shown in Table 2.1

Table 2.1: Comparison of Colorimetric and Potentiometric Determinations of F^-

Sources of fluoride	Amount of fluoride added to basal medium: mg $F^-.l^{-1}$ (or µM)	Methods of F^- determination colorimetric (mg $F^-.l^{-1}$)	potentiometric (µM)
NaF	0.0	0.7*	MR < 1.0**
	0.013 (0.72 µM)	0.9*	MR < 1.0**
	0.137 (7.20 µM)	1.4*	6.8**
	1.37 (72.00 µM)	1.8*	72.0**
	13.70 (720.00 µM)	OTR	720.0**
NaFa	0.0	0.0+	MR < 1.0**
	0.475 (10.0)	0.7+	MR < 1.0**
	0.950 (50.0)	1.4+	MR < 1.0**

Data entered are averages of triplicates.
For potentiometric method, the F^- -specific electrode was previously calibrated with NaF standards made in 10 mM phosphate buffer, pH 6.8(**).
Readings of F^- obtained from calibration curves prepared from NaF standards made up in distilled water (*), or basal medium (+) as shown in Fig. 2.2.
OTR: Outside the range of sensitivity of the method used (Bellack & Schouboe, 1958).
MR: F^- reading on specific ion meter (Orion Model 407) less than 1.0 µM F^- which is the lowest limit of detection using the potentiometric method (Orion Research Incorporated, 1973).

FIG. 2.2 CALIBRATION CURVES OF NaF STANDARDS: COLORIMETRIC METHOD



NaFa standards were made up in basal medium and the concentration of fluoride determined using both methods. Results given in Table 2.1 show that a significant amount of fluoride was detected in the NaFa standards using the colorimetric method whilst this was not detected by the potentiometric method. This suggests that the NaFa standards were contaminated with F^- (n.b. most commercial NaFa does contain variable amounts of F^-) or that NaFa-F may be able to react with the $ZrO^=$ - SPADNS reagents (used in colorimetric method) to some extent. The latter possibility may explain why F^- was not detected by the potentionmetric method.

The colorimetric method is not adequate for determining F^- concentration greater than 72 µM in the basal medium which contains mineral ions ($PO_4^=$, Mg^{++}, Cl^-, Ca^{++}) which interfere with the colour change of the complex (Bellack & Schouboe, 1958). The potentiometric method is less sensitive to interference by other ions, and thus the potentiometric method of F^- determination was used throughout this project for measuring F- concentration greater than 1.0 µM.

2.3.2. Diversity of NaFa-Degrading Micro-organisms

NaFa enriched medium was inoculated with soils sampled from six districts of the South Island where NaFa had been applied. Eight genera of bacteria showing ability to defluorinate NaFa were isolated and listed in Table 2.2. However, a zero entry is not necessarily an indication of the absence of bacteria in those soils.

Selection of bacterial isolates was based on morphological appearances of the bacterial colonies on nutrient agar plates: texture, size, shape and colour of the colony. Bacterial cells were then examined for motility, and Gram-stained. Consequent on the deliberately selective method, some degree of diversity was observed (Table 2.3).

TABLE 2.2 Occurrence of soil micro-organisms capable of defluorinating Na fluoroacetate. Soil samples (except Heathcote River) taken from loading area of airstrip runways. Enrichment cultures were grown on mineral salts medium containing 20 mM-NaFa. Triplicate experiments

Micro-organisms detected	Soil Sample							
	Cape Foulwind	Oweka[1]	Hukarere	Mawheraiti	Taiko[2]	Waipuna	Inangahua	Heathcote River (mud)
Psuedomonas sp	+++	+++	+++	+++	+++	+++	+++	+++
Bacillus subtilis	++	++	0	++	+	+	0	0
Aeromonas sp	+	+	0	+	+	+	0	0
Actinobacillus sp	+	+	0	+	+	+	0	0
Proteus sp	0	+	0	0	0	0	0	0
Lactobacillus sp	0	+	0	0	++	0	0	0
Staphylococcus sp	0	+	0	0	0	0	0	0
Citrobacter sp	0	+	0	0	0	0	++	0
Fungi (various)	+	+	+	+	+	+	+	+

Relative abundance of each species denoted thus: 0, +, ++, +++.

1. Previous history of dairy farming and outbreak of mastitis.
2. Previous hsitory of cattle grazing.

TABLE 2.3 Diversity of NaFa-Degrading Micro-organisms: Bacteria and Their Relative Defluorinating Activity

Bacterial isolates	Gram stain	Appearance of Colony and Cells	F^- (mM) released into medium	% defluorination of NaFa
Pseudomonas sp	-ve	creamy white colony. Motile rods.	20	100
Aeromonas sp	-ve	smooth and translucent. Motile rods.	1.1	5.5
Citrobacter sp	-ve	same as above	0.12	0.6
Proteus sp	-ve	Moist, clear and raised. Motile rods.	0.56	2.8
Actinobacillus sp	-ve	White colony firmly held to agar surface. Small rods, non-motile.	0.36	1.8
Bacillus subtilis	+ve	Flat thin colony with serrated edge. Large rods. Motile.	6.4	32.0
Staphylococcus aureus	+ve	Coccus. Colony assumed a pinkish gold colour.	0.36	1.8
Lactobacillus	variable	White colony. Pleomorphic.	0.12	0.6

Pure culture of each bacterial isolate (equal inoculum density) grown in 20 mM NaFa medium, pH 6.8 at 25°C for 7 days. Data entered are averages of triplicates.

The species of *Pseudomonas* isolated was neither *P. auruginosa* nor *P. fluorescens*. Standard biochemical tests showed that the isolate of *Proteus* was not one of the known pathogens commonly described: *Pr. vulgaris*, *Pr. rettgeri* nor *Pr. mirabilis*. Other bacteria isolated were *Actinobacillus* sp, *Staphylococcus aureus*, *Citrobacter* sp and a pleomorphic Gram-variable species which was tentatively placed under the genus, *Lactobacillus*. These latter bacterial isolates were less frequently detected, and occurred in areas which had been previously farmed. *Pseudomonas* sp, and *Bacillus subtilis* were more commonly found.

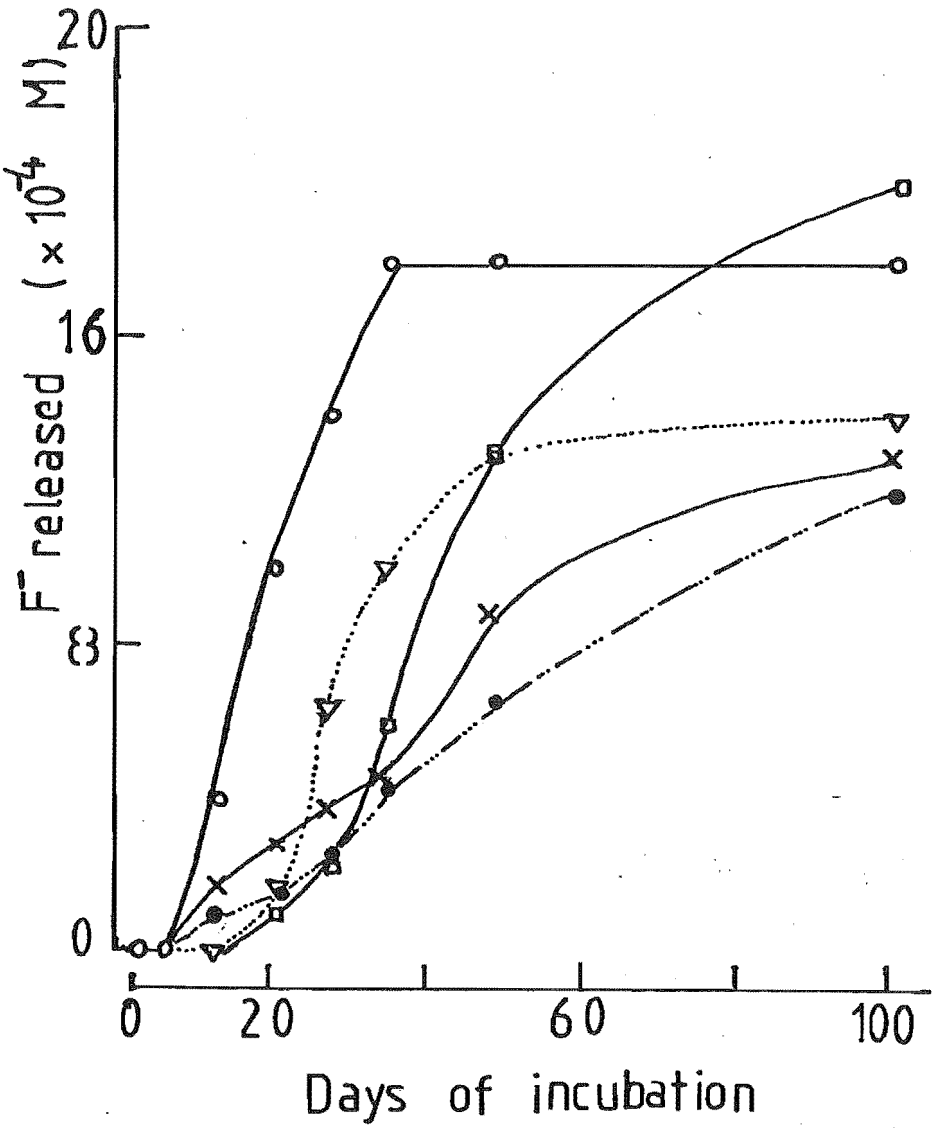
Other micro-organisms isolated include fungi - *Fusarium solani* (Mart.) Sacc., *F. oxysporum* (Schlecht), *Acremonium strictum* W. Gams, species of *Penicillium* and *Aspergillus*; and unicellular algae, one of which was isolated and identified as *Chlorella* sp by Dr G. McRaild (University of Canterbury, Christchurch).

The relative defluorinating activity of bacterial isolates was tested by growing each in 20 mM NaFa medium at 25°C for 7 days, and measuring the release of F^- into the medium. Results are given in Table 2.3. *Pseudomonas* sp, and *B. subtilis* were more active, *Lactobacillus* sp and *S. aureus* showed comparatively poorer growth but were able to defluorinate NaFa to a limited extent. The presence of these organisms in those soils examined suggests the existence of a biological potential for decomposition of NaFa.

2.3.3. Persistence of NaFa in Soils

Defluorinating activity of the soils from which these organisms were isolated were examined using freshly collected soils. As shown in Figure 2.3 no free F^- was detected for the first 5-10 days of incubation, and a progressive release of F^- was observed for the next 90 days of incubation. At the end of the incubation period, only 10% of NaFa -F initially applied to the soil was detected in the form of free F^- for Taiko and Waipuna samples, 9% for Cape Foulwind sample, 6 - 8% for Hukarere, Mawheraiti and Oweka samples. The pattern of F^- release over the period was characteristically sigmoid for each soil sample examined. No F^- was detected from NaFa-treated soils which had been previously sterilised by autoclaving. From F^- release curves, the Oweka and Cape Foulwind soils were

FIG 2.3: RELEASE OF SOLUBLE F^- FROM SOME SOUTH ISLAND SOILS TREATED WITH NaFa



Soils (250 g) were mixed with NaFa (2.5 g), incubated at 25°C. 25 g sample was withdrawn at intervals, shaken with 125 ml basal medium for 30 min, and soluble F^- measured. No soluble F^- was detected in sterilised NaFa-treated soils.

SOILS SAMPLED FROM

- CAPE FOULWIND
- WAIPUNA, TAIKO
- HUKURERE
- OWEKA
- MAWHERAITI

more active in degrading NaFa as indicated by steeper slopes. Further incubation of soil solution (devoid of soil particles) showed further release of F^- into unsterilised medium (Table 2.4) but not in sterilised medium. This suggests that soil solutions contained NaFa which was subsequently degraded by factors which were thermo-labile (biological) on incubation. Soil solutions prepared from soil sampled immediately after it was mixed with NaFa contained no F^- until it was further incubated. The concentration of F^- in the soil solution after incubation gave an estimation of NaFa extracted from NaFa treated soils and this varied from 7.5-14% of NaFa initially applied to the soil as shown in Table 2.4.

The role of micro-organisms in soils in the decomposition of NaFa was studied further by following F^- release and growth of microbial population. In this study, triplicates of 10 ml aliquots of 20 mM NaFa medium in 100 ml conical flasks were inoculated with 0.2 g of air dried finely-ground soils from Oweka. The soil samples from Oweka differed in their previous exposure to NaFa, as given in Table 2.5. There was no great variation in the defluorinating activity of these samples. Samples from sites with relatively richer organic content (site 5 and 7) showed higher defluorinating activity. Lowest activity was detected from sites which were relatively uncontaminated previously. Bacteria were present in all the soil samples. *Penicillium*, *Aspergillus* and *Fusarium* species were also detected. *Mucor* sp., was found in soils sampled from some sites of Oweka, was not detected in those sites with a history of NaFa contamination, except at site 1 (bait loading area).

The relationship of microbial growth to decomposition of NaFa was shown in Figure 2.4. A lag in microbial growth was observed for culture flasks inoculated with soil from site 3 (contaminated area) and site 6 (indirectly contaminated), but not from site 5 (fertilized area which had not been previously contaminated with NaFa). In all culture flasks there was an initial lag in NaFa defluorination. Following the lag phase was the exponential stage of microbial proliferation and defluorination of NaFa. The pattern of F^- release followed closely that of microbial growth. The initially observed lag was abolished on successive

Table 2.4 Further defluorination of NaFa in unsterilised soil solutions.

Day on when soil solutions prepared.	Concentration of F^- in soil solutions prepared from :											
	Oweka		Hukarere		Waipuna		Mawheraiti		Cape Foulwind		Taiko	
	F^-_t	F^-_{tt}	F^-_t	F^-_{tt}	F^-_t	F^-_{tt}	F^-_t	F^-_{tt}	F^-_t	F^-_{tt}	F^-_t	F^-_{tt}
0	0	2.0	0	2.3	0	2.8	0	1.7	0	1.5	0	2.7
3	0	4.0	0	10.0	0	3.6	0	1.8	0	2.2	0	2.4
10	0	2.7	0.11	2.2	0	3.6	0.28	1.8	0	2.0	0	3.5
17	0.18	3.0	0.15	4.6	0.11	3.8	0.28	3.4	1.0	2.6	0.13	3.5
24	0.64	10.0	0.26	6.4	0.35	5.8	0.38	6.2	1.3	6.4	0.30	6.4
31	1.0	8.4	0.44	6.2	0.60	8.6	0.46	5.4	1.8	4.9	0.60	10.0
45	1.3	3.0	0.66	5.2	1.3	4.0	0.90	7.0	1.8	2.2	0.6	8.8
97	1.4	1.6	1.2	2.3	2.0	2.0	1.3	1.4	1.8	1.8	2.0	2.0

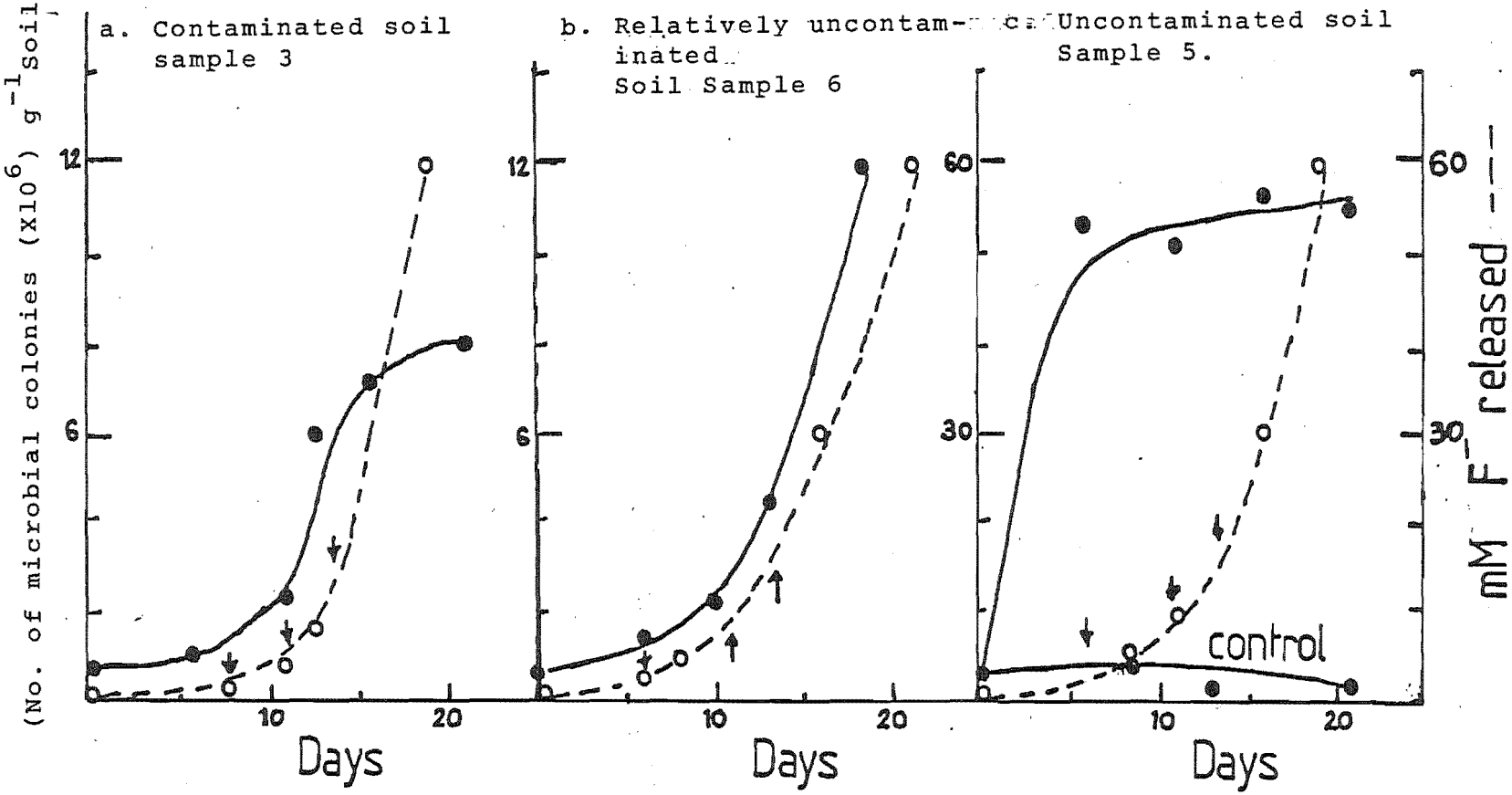
Soil solutions were prepared as described in Fig 2.3. F^-_t = initial concentration of F^- of soil solution, and F^-_{tt} = concentration of F^- of the soil solution after further 10 days incubation at 25°C. F^- determined were in duplicate. Further increase in F^- concentration of sterilised soil solutions was not detected after subsequent incubation.

TABLE 2.5 Defluorinating ability of soil samples taken from sites around the Oweka airstrip. Enrichment cultures grown in mineral salts medium containing 20 mM-Na fluoroacetate. Concentration of F^- in culture medium assayed after 11 days incubation at 25°C. Triplicate Experiments.

Sample No.	Site	F^- conc (mM)	NaFa degradation (%)	RELATIVE ABUNDANCE OF MICRO-ORGANISMS: Bacteria	*Fusarium* sp	*Mucor* sp	*Penicillium* sp	*Aspergillus* sp	*Algae**
1	Bait loading area	15	75	+++	++	+	+	+	+
2	Relatively uncontaminated area	13	65	+++	++	+	+	+	+
3	Bait loading area	14	70	+++	++	0	+	+	+
4	Bait preparation area	14	70	+++	++	0	+	+	+
5	Uncontaminated area treated with superphosphate	17	85	+++	++	+	+	+	+
6	Drainage ditch in area 1	12	60	+++	0	0	+	+	+
7	Humus layer from area 4	17	85	+++	++	0	0	0	+

Relative abundance of each species denoted thus 0, +, ++, +++.
*Species of NaFa-tolerant *Chorella* and *Chlamydomonas* appeared after 18 days.

Fig 2.4 Relationship of microbial growth & NaFa defluorination



NaFa medium (pH 6.8) was inoculated with soils from Oweka, incubated at 25°C. Microbial population was enumerated, F^- released determined and NaFa (20 mM) replenished at intervals (↑). Results are averages of triplicates.

TABLE 2.6 Effect of NaF on Defluorination of NaFa by Soil Microbial Population

NaF (mM) initially applied	CONCENTRATION OF F^- (mM) IN CULTURE FLASKS INNOCULATED WITH SOILS FROM OWEKA SAMPLED AT:								
	Site 3			Site 5			Site 6		
	R_0	R_1	R_2	R_0	R_1	R_2	R_0	R_1	R_2
Nil	Nil	4.0	4.0	Nil	8.0	8.0	NIl	3.0	3.0
10	9.2	14.0	4.8	9.9	16.0	6.1	10.0	11.0	1.0
30	25.0	31.0	6.0	27.0	32.0	5.0	22.0	23.0	1.0
50	46.0	47.5	1.5	49.0	49.0	0.0	39.0	40.5	1.5
80	72.0	73.0	1.0	69.0	69.0	0.0	60.0	60.0	0.0
100	92.0	92.0	0.0	88.0	88.0	0.0	-	-	-

Note: 1. Soil suspensions (pH 6.8) were incubated at 25°C for 12 days. F^- then measured. Data are averages of duplicates

R_0 - culture flasks without NaFa

R_1 - Culture flasks with 20 mM NaFa

R_2 - NaFa degraded (mM), difference between R_0 and R_1

application of 20 mM NaFa. When growth had reached a stationary phase, F^- release continued exponentially as seen from Figure 2.4a and 2.4c indicating that defluorinating potential was retained even when there were factors limiting microbial proliferation.

2.3.4. Inhibitory Effect of NaF-F on Defluorination of NaFa

One of the breakdown products of NaFa was inorganic F^-, an element toxic to plants at high concentrations. The effect of NaF on soil microbial defluorination of NaFa was studied. Soil inocula were taken from three sites of Oweka. As shown in Table 2.6, defluorination of NaFa was reduced and the inhibition increased with increasing concentration of NaF applied to the soils (0.2 g sample) in solution. A stimulation was observed for soil inoculum from site 3 at 30 mM NaF but at higher concentrations, inhibition was observed. The significance of the apparent stimulation could not be assessed as the sample size was too small.

More F^- was lost to detection by the F-specific electrode as more NaF was applied to the soil after 12 days incubation. The amount lost varied from 1-8% at low NaF concentration range to 8-25% of initial NaF applied to the soil at the higher concentration range. This highlights the problem of F^- adsorption by soils.

2.4 DISCUSSION

2.4.1. Diversity of NaFa-degrading Microorganisms.

The term 'defluorination' is used synonymously with biodegradation or decomposition throughout inasmuch as it denotes a detoxifying mechanism. The toxicity of NaFa depends on intact carbon-fluoride bond. Cleavage of the bond (defluorination) renders the molecule non-toxic. Hence the above terms are used interchangeably. The C-F bond of NaFa is remarkably stable: it could withstand boiling in H_2SO_4 without F^- being released, and boiling in alcoholic KOH released only 50% of F^- (Saunders, 1972). There were reports

that aqueous NaFa solution was unstable (Chenoweth, 1949; Harrison *et al.*, 1951; Hilton *et al.*, 1969). Chenoweth cited an observation made by Albaum that the rate of deterioration in toxicity of methyl fluoroacetate solution was reduced when stored in a refrigerator. Harrison *et al.*, (1951) reported that there was a progressive loss of F^- in aqueous NaFa solution but not in saline solution over 133-182 days of storage. Kalnitsky & Barron (1947) reported that NaFa solution remained stable when stored at 3-5°C for a month. Implicit in the temperature dependence of stability of NaFa solution at physiological conditions is the role of biodeterioration. Biological breakdown of NaFa was first reported in 1965, about 23 years after the compound has been used world-wide as a pesticide (Kelly, 1965; Tonomura *et al.*, 1965; Goldman 1965). The only bacteria reported to be able to defluorinate NaFa at that time were species of *Pseudomonas*. In the present studies, eight genera of NaFa-degrading bacteria, and a few species of fungi were isolated from soils previously exposed to NaFa. The different bacterial isolates showed varying ability to defluorinate NaFa and use it as a carbon source for growth. Among them, *Pseudomonas* and *Bacillus subtilis* were most active and commonly found; and relatively less active were *Aeromonas, Actinobacillus, Citrobacter, Staphylococcus aureus* and another tentatively classified as *Lactobacillus*. The latter isolates were detected from areas previously farmed. It is probable that these could have been brought to the areas by animals and man. Likewise *Mucor* sp. found in some soils sampled from Oweka could have been similarly introduced. The other fungi isolated were *Fusarium solani, F. oxysporum,* species of *Penicillium* and *Aspergillus,* and *Acremonium strictum*. The relative ability of these fungi to defluorinate NaFa will be examined later. It is possible that an even greater diversity of NaFa-degrading micro-organisms could exist if different enrichment media and culture conditions had been used. Culture conditions used were selective for aerobes. Not every isolate that appeared on dilution plates was screened for its defluorinating ability. Related to this is the question as to whether NaFa has any adverse effects or otherwise on the nitrogen status of soils, and the balance of autotrophic microbial populations. It has been reported that nitrogenase activity of *Gloeocapsa* sp was depressed by fluoroace-

tate (Tozum *et al.*, 1977; Gallon *et al.*, 1978).

The variability of bacterial isolates in defluorinating NaFa may be a reflection of the influence of some other limiting factors rather than an indication of limited potential of these lesser active isolates tested under the conditions described. This could be true in the case of *Actinobacillus* sp., *S.aureus*, and *Lactobacillus* sp. which have fastidious nutrient requirements. That environmental factors such as carbon and nitrogen status, and physical variables could influence the defluorinating activity will be examined and discussed later.

2.4.2. Persistence and Biodegradation of NaFa

The presence of these NaFa-degrading micro-organisms in all the soils examined suggests the potential of these soils for defluorination of NaFa. The persistence of NaFa in these soils was examined. It was found that only 7 - 8% of NaFa-F appeared as water-soluble free F^- over 98 days of incubation. The pattern of F^- release from all the unsterilised NaFa-treated soils was characteristically sigmoid and this was taken to be indicative of biological rather than physical deterioration (Audus, 1964) since the latter would yield a straight line. The method used to extract F^- was not exhaustive. No attempt was made to extract inorganic F^-, which was complexed to other metals such as Al^{+++} or absorbed to soil particles, by ashing and acidified distillation as recommended by Brewer (1965). The method presently used entailed shaking for 30 mins, to extract water-soluble F^- from soils. From analysis of tropical forest soils - clay, silt, sandy loam, and clay loam of pH range 4.4 - 8.5, Hall & Cain (1972) reported that maximum water-soluble F^- constituted only 0.1 - 5.0% of total fluorine content, over 80% was found as acid-labile or complexed inorganic F^- and a small percentage in organic form. On the basis of their findings, the amount of water-soluble F^- extracted (7 - 10%) from soils treated with NaFa would not be too small and it could be deduced that about 80 - 100% of NaFa initially applied was defluorinated, with most of F^- complexed or adsorbed and rendered undetectable by F^--specific electrode.

Recently the use of the F^--specific electrode has increased

in popularity for its simplicity, reliability in yielding rapid and reproducible results. It has been used to measure F^- concentration in vegetation, soils, and animal tissues such as bone and teeth (Larsen & Widdowson, 1971; Peters & Baxter, 1974; Cooke *et al.*, 1976a; McClenahen & Schultz, 1976). In the present studies it was found that the colorimetric method of F^- determination lost its sensitivity to F^- in the presence of other ions, (Bellack & Schouboe, 1958), whereas the potentiometric method was rapid and less sensitive to interference by other ions provided the electrode was calibrated with F^- standards made up in solution of ionic composition similar to the sample to be measured. Hence the F^--specific electrode was used throughout the studies. Determination of F^- released into the soil or culture medium was used for estimation of NaFa degradation. No attempts were made to analyse NaFa residues. Although several standard procedures have been developed for detection of fluoroacetate using a colorimetric method (Hutchens & Kass, 1949), paper chromatography (Bergman & Segal, 1956; Vickery *et al.*, 1973), gas-liquid chromatography (Ward, 1973; Hall & Cain, 1972), any attempt to measure NaFa residues would require re-appraisal of the above methods and the adequacy of each method and modification to the materials studied. It is anticipated that this would be a major time-consuming task, and in view of the other aspects to be studied, such attempts were not made. Furthermore, the measurement of F^- (1μM to 100 mM) as an estimation of NaFa degradation in a defined system as presently applied is simpler, faster and adequate for the present purpose.

The persistence of NaFa in garden soils was studied by David & Gardiner (1966), by assaying residual toxicity to aphids which infested plants grown on NaFa-treated soils. They reported that steam-sterilised NaFa-treated soils remained toxic after 11 weeks whereas unsterilised NaFa-treated soils lost the toxicity within that period. Since reports of successful isolation of bacteria that could cleave the C-F bond of NaFa, the role of micro-organisms in the decomposition of NaFa has been recognised. In the present work, the relationship of microbial proliferation and NaFa degradation was studied using soil as inoculum. A close correlation was found between F^- released (or NaFa degraded) and microbial growth. A similar

relationship was demonstrated with fluoroacetamide as the sole C-source and a pure culture of bacteria (Kelly, 1965). Most of the earlier reports on instability of NaFa solution at physiological conditions could be attributed to microbial activity. Observation was made during the course of present work on stability of aqueous solution during storage at room temperature (18-22^{o}C). Free F^- was not detectable in NaFa solution previously sterilised, then stored for 2½years, whereas unsterilised solution rapidly became turbid and over 20% of NaFa-F was found as free F^- after 2 days storage under the same conditions.

2.4.3. Effect of NaF on Soil Microbial Defluorination of NaFa

In preceding discussion, it was suggested that most of F^- from NaFa breakdown in soils was complexed or adsorbed to soil particles, and therefore was not detected by the F^--specific electrode. The adsorption and/or complexing potential of the soils was tested by applying NaF (0-100 mM) solutions to the soils (0.2 g' 20 ml^{-1}). After 12 days incubation, 1 - 25% of the initial NaF applied was not detectable by using the F^--electrode indicating that F^- must be complexed and/or adsorbed to soil minerals or particle surfaces. The amount of F^- complexed or adsorbed depends on the initial concentration of NaF and increased with increasing NaF concentration.

When present in sufficiently high concentration, F^- was found to be inhibitory to defluorination of NaFa in soils, probably via a feedback mechanism. Inhibition increased with increasing F^- concentration which may suggest a competitive type of inhibition at the site of entry to bacterial cells. The effect of F^- will be examined at the cellular and enzymatic level.

CHAPTER THREE

EFFECT OF ENVIRONMENTAL FACTORS ON BIODEGRADATION OF NAFA

3.1 INTRODUCTION

The persistence of pesticides is influenced by enviornmental factors such as physical weathering and/or biological processes. Because of the extraordinary physical and chemical stability of NaFa, its detoxification would be achieved mainly via biological means. Although biodegradability of NaFa had previously been demonstrated using bacteria (Kelly, 1965; Goldman, 1965; Tonomura *et al.*, 1965), the effect of environmental factors on the biological agents and the process had not been investigated. Consequently the following studies on the influence of such environmental variables as pH, temperature and nutrient status on the organism and NaFa-degradation were carried out. In all experiments, with one exception, fungi were used in preference to bacteria despite the attractive attribute of the latter in rapidity of growth.

3.2 MATERIALS AND METHODS

3.2.1. Organisms, Culture and Maintenance

The organisms selected for study were all isolated from soils from earlier work with enrichment cultures: *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht., *Acremonium strictum* W. Gams, *Penicillium* sp., and *Pseudomonas* sp. Cultures of fungi were maintained on malt-extract agar, potato-dextrose and potato-sucrose agar slopes. *Pseudomonas* sp. was maintained in liquid NaFa mineral salts medium or nutrient agar slopes. All growth experiments were carried out in liquid NaFa mineral salts medium of a composition as previously described. (2.2.3) Unless otherwise stated, all media were sterilised by autoclaving at 120°C for 15-20 mins. Medium with urea as nitrogen source was filter-sterilised. Medium buffered with 10 mM phosphate buffer was also filter-sterilised, using 0.45 µm microfilter.

3.2.2. Preparation of Fungal Spore Inoculum and Mycelial Inoculum

Fungal spores were harvested from 7-day old cultures on slopes or plates, with sterile distilled water. Mycelial debris were removed by filtering the preparation through a double layer of sterile muslin cloth. Density(spores ml^{-1}) of the spore suspension was determined using a haemacytometer and standardised

for each set of experiments. Only freshly prepared spore suspensions were used. Mycelial inocula were prepared aseptically from discs cut with a 10 mm diameter cork borer from 7-day old plate cultures.

3.2.3. Determination of Fungal Growth and NaFa Degradation

Fungal growth was determined by measuring the dry weight of mycelium produced. Mycelium was collected by filtering the culture through pre-weighed Whatman No 1 filter paper, washed several times with distilled water, then dried at 78°C for 24 hours. When cooled to room temperature, the mycelium was weighed. Because only small amounts of mycelium were produced in some experiments, mycelium from triplicates was pooled for total weight determination and average weight per replicate was then calculated. Variations due to measurement error or inherent variability in growth was estimated from the coefficients of variation computed from 5 - 7 triplicates of mycelium yielded from the same treatment.

NaFa degradation was determined by measuring the amount of F^- released into the culture medium using the F^--specific electrode.

3.2.4. Identification and Quantitative Determination of Glycolate

Glycolate was identified and determined using the colorimetric method of Lewis and Weinhouse (1957). Glycolate produces a red-violet compound when heated with 2:7-hydroxynapthalene, and color intensity is proportional to glycolate content. The color produced was read on an EEL colorimeter using a 530 nm filter. A calibration curve was prepared using glycolate standards made up in distilled water.

3.2.5. General Procedure

Aliquots of 10-ml or 50-ml of mineral salts medium were dispensed into 100-ml or 250-ml conical flasks and autoclaved. The flasks were then inoculated with spore suspension and incubated without shaking for 5 days at 25°C unless otherwise mentioned. At the end of the experiment, concentration of F^- in each culture flask was measured to determine NaFa degradation, subsequently the mycelium was harvested.

3.3. RESULTS

3.3.1. NaFa as the Sole Carbon Source for Growth

Fungi isolated from soils previously exposed to NaFa were examined for their ability to degrade and utilize NaFa as the sole carbon source for growth. Triplicates of 50 ml aliquots NaFa medium were inoculated with fungal spores and incubated for 5 days at 25°C. As shown in Table 3.1, spores of *Penicillium* sp., and *Aspergillus* sp., germinated but further growth was not evident and the concentration of free inorganic F^- in the culture medium was negligible. The yields of growth of *Fusarium solani, F. oxysporum* and *Acremonium strictum* in medium with NaFa as the sole carbon source were approximately 1.6 mg and 0.78 mg mycelium respectively per culture flask respectively for both *Fusarium* species and *A. strictum*. There was a significant release of F^- into the culture medium in which the *Fusarium* species and *A. strictum* grew, and a concentration of 3.2 and 1.2 mM F^- was recorded at the end of the incubation period for the former and latter species of fungi respectively. The F^- level in uninoculated NaFa medium was negligible, showing that there was no spontaneous defluorination of NaFa. In control media without any carbon source but inoculated with spores of *F. solani*, some growth was observed, about 10% of the yield in medium with NaFa. The variability of growth of *F. solani* under the same conditions as above was determined from 5 triplicates of 50-ml samples and 7 triplicates of 100-ml sample inoculated with 3 agar discs of mycelium. The coefficient of variation of mycelium yeild, on a dry weight basis, was 12.7% and 9.2%, for the spore- and mycelium-inoculated cultures respectively. These values could be used to estimate general variability and significance of difference in mycelial weights obtained from various treatments in the above and following experiments.

3.3.2. Effect of Nitrogen Sources

The effect of different nitrogen sources (NO_3^- or NH_4^+ or urea) present singly in a medium with NaFa as the sole carbon source, on the growth and defluorination of NaFa by *F. solani, F. oxysporum* and *A. strictum* was studied. Triplicates of 10 ml aliquots NaFa medium inoculated with spores and incubated at 25°C for 5 days. The concentration of nitrogen applied was 212 mg ammonium-N, 236 mg nitrate-N, or 540 mg urea-N per litre of NaFa medium. Results presented in Table 3.1 showed that *F. solani* and

Table 3.1 Effect of Carbon and Nitrogen Sources on Growth and NaFa defluorination by Various Fungi

Sources of carbon ($0.2\ g\ NH^{+}_{4}\text{-}N\ l^{-1}$)	FUNGAL SPECIES									
	F. solani		*F. oxysporum*		*A. strictum*		*Penicillium* sp.		*Aspergillus* sp.	
	myc wt (mg)	F^- released (mM)	myc wt (mg)	F^- released (mM)	myc wt (mg)	F^- released (mM)	myc wt (mg)	F^- released (mM)	myc wt (mg)	F^- released (mM)
20 mM NaFa	1.62	3.2±0.4	1.56	2.9±0.3	0.78	1.2±0.1	sparse	0.13	sparse	0.15
10 mM NaFa + 10 mM glucose	8.20	6.0±0.0	7.5	5.1±0.5	7.65	5.7±0.4	3.8	1.0±0.0	2.7	0.61±0.05
10 mM NaFa + 10 mM acetate	3.07	4.3±0.2	3.40	4.2±0.1	2.77	3.5±0.6				
Sources of N (20 mM NaFa)										
$0.2\ g\ NO_3\text{-}N\ l^{-1}$	0.6	1.7±0.1	0.63	1.0±0.0	0.87	0.9±0.0				
$0.5\ g\ urea\text{-}N\ l^{-1}$	0.72	2.1±0.0								

Dry weight of mycelium (myc wt) expressed as average of triplicate culture flasks, incubated at 25°C for 5 days. Inorganic F^- released is average ± standard deviation.

F. oxysporum grew best in medium with ammonium-N whereas *A. strictum* showed no marked preference for the forms of nitrogen provided. The yield of mycelium in ammonium-N medium was about twice that in nitrate-N or urea-N in the case of *F. solani*. The effect of urea was not tested on *F. oxysporum* and *A. strictum*. Highest percentage of NaFa degraded, as estimated by the amount of F^- released into the culture medium, was found in cultures with ammonium-N as the nitrogen source: 16%, 14,5%, and 6% by *F. solani*, *F. oxysporum* and *A. strictum* respectively. In nitrate-N medium, the amount of NaFa degraded was 8.5% 5%, and 4.5% by the fungi listed in the above order. The results thus showed that NH_4^+-N was the preferred form of nitrogen source for both the growth and defluorination of NaFa by *F. solani* and *F. oxysporum* while the effects of NH_4^+ and NO_3^- on *A. strictum* were not significantly different.

To give an indication of the efficiency of fungal utilization of NaFa economic coefficient is calculated. The economic coefficient is defined here as the ratio of growth (mycelial weight in mg) to the amount of carbon made available from defluorination of NaFa. It is assumed that for every mole of NaFa defluorinated, two moles of carbon were made available to the fungus. Highest economic coefficient was found in ammonium-N and lowest in urea-N for *F. solani* whilst it remained the same for *F. oxysporum* and *A. strictum* in both ammonium-N and nitrate-N medium.

The effect of increasing concentration of ammonium-N on the growth and defluorination of NaFa by *F. solani* was also studied in triplicates of 10 ml samples inoculated with spores. Concentration of ammonium-N ranged from 0.2 g to 21.2 g l^{-1}. As shown in Table 3.2, no significant difference in growth was observed within the concentration range 0.2 g - 0.3 g l^{-1}, whereas NaFa degraded increased from 35% to 60%. Growth was slightly increased at 0.4 and 1.0 g l^{-1} while NaFa degraded decreased from 75% at 0.4g l^{-1} to 32% at 1.0 g l^{-1}. At 21.2 g l^{-1}, both growth and NaFa degraded were consideraby reduced. While growth was relatively unaffected at the lower concentration range of 0.2 g - o.4g l^{-1}, NaFa degraded increased proportionally with increase in ammonium-N concentration so that a reduction in economic coefficient was observed.

TABLE 3.2: Concentration Effect of $(NH_4)_2SO_4$ on Growth and Deflourination of *F.solani*

Concentration of NH_4^+-N ($g\ l^{-1}$)	myc wt (mg)	F^- (mM)	Economic Coefficient
0.2	1.2	6.8	0.73
0.22	1.2	7.02	0.71
0.3	1.23	12.0	0.43
0.41	1.57	15.0	0.44
2.12	1.42	6.4	0.85
21.2	0.08	0.92	0.36

F. solani was grown in 20 mM NaFa medium and incubated for 5 days at 25°C. F^- released mycelial yield (dry wt) then measured. Results are averages of triplicates.

3.3.3 Effect of pH

The effect of pH on the defluorination of NaFa and growth of *F. solani* was studied in triplicates of 10 ml aliquots of 20 mM NaFa medium inoculated with spores and incubated as before. Growth medium was buffered with 10 mM phosphate buffer from pH 5.0 - 7.8. As shown in Figure 3.1, the optimal pH for both growth and defluorination of NaFa was found to be 5.8, at which pH the mycelial yield averaged 1.65 mg per culture flask and 65% of the initially applied NaFa was defluorinated. While growth was relatively unaffected between pH 5.8 to 7.2, defluorination was highly sensitive to pH lower or higher than 5.8. Economic coefficient was lowest at pH 7.8 (0.6) and highest pH 5.0(0.8).

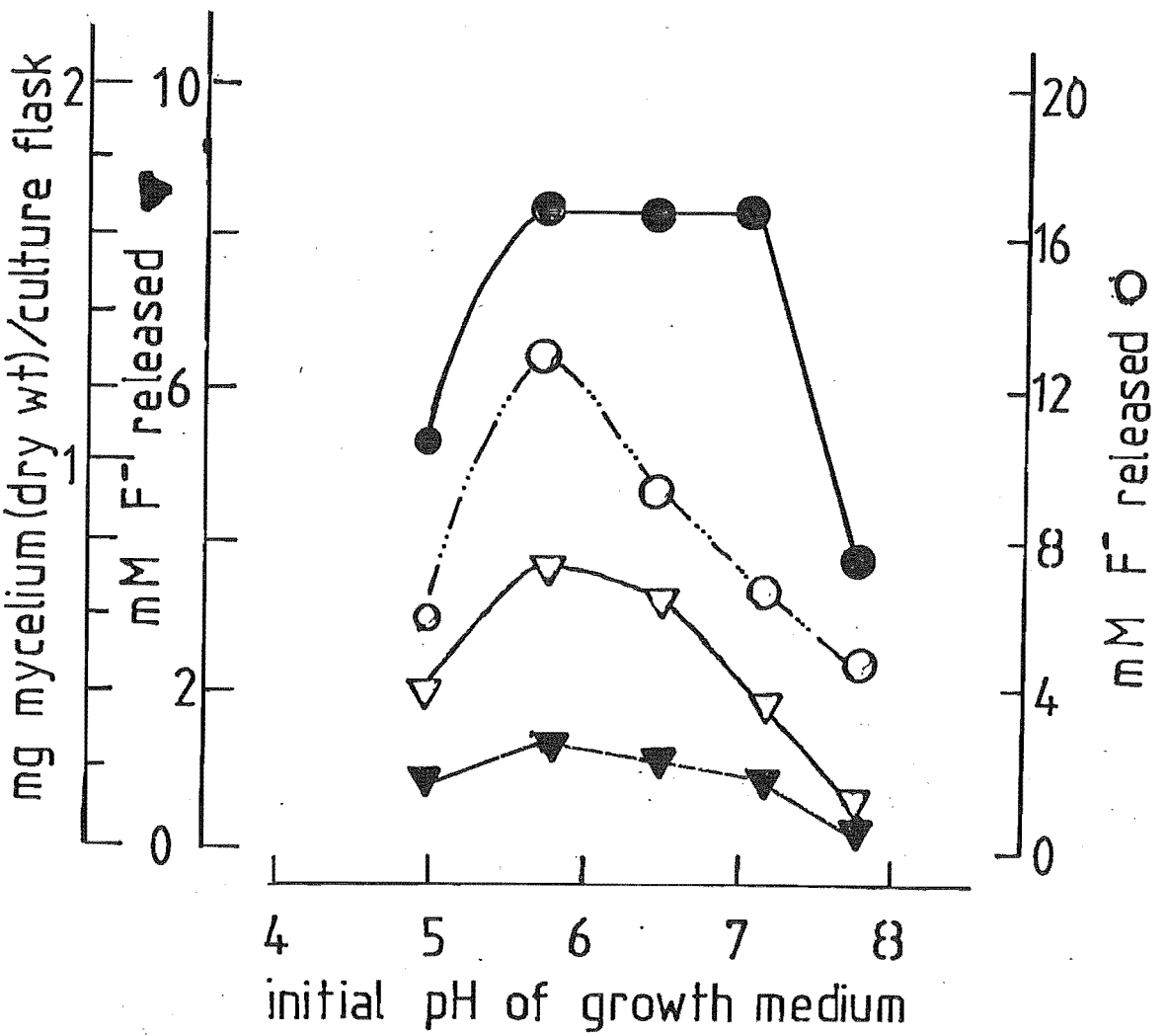
Similar experiment was carried out using *A. strictum* in triplicates of 50 ml aliquots of 20 mM NaFa medium inoculated with spores. Fig 3.1 shows that NaFa defluorination was relatively unaffected within the pH range, 5.0 - 7.2, wherein about 5 - 6% of NaFa initially applied was defluorinated. Growth was more sensitive to external pH with an optimum at pH 5.8 (0.75 mg per culture flask) and minimal growth was observed at pH 7.8 (0.12 mg per culture flask). Ecomomic coefficient was lowest at pH 7.8 (0.13) and highest at 6.5 (0.5).

3.3.4 Effect of Temperature

Triplicates of 50-ml aliquots 20 mM NaFa medium inoculated with spores of *F. solani* were incubated at 4°C, 10°C, 20°C, 25°C, and 30°C, for 5 days without shaking. Growth was not evident at 4°C and the concentration of F^- in the culture medium was similar to that in uninoculated controls. As shown in Figure 3.2 both growth and defluorination of NaFa increased with increase in the temperature of incubation. Thus the response of growth of the fungus and its defluorination of NaFa was similar and it follows that economic coefficient at all temperatures is constant within the range examined.

The experiment was repeated with 10 replicates of 150 ml aliquots of 20 mM NaFa medium supplemented with o.05% glucose (w/v) in 1l Roux bottles, inoculated with 5 agar discs of mycelium of *F. solani*, and incubated for 10 days at 10°C, 20°C, 25°C, 30°C or 40°C.

FIG 3.1: EFFECT OF pH ON FUNGAL GROWTH AND ON DEFLUORINATION OF NaFa



NaFa media of different pH (10 mM phosphate buffer) were inoculated with fungal spores, incubated at 25°C for 5 days. F^- released and mycelial yield determined. Results are averages of triplicates

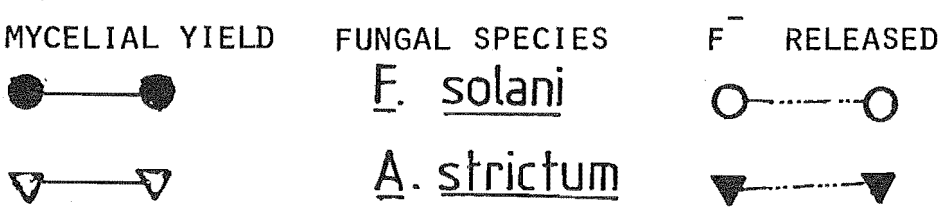
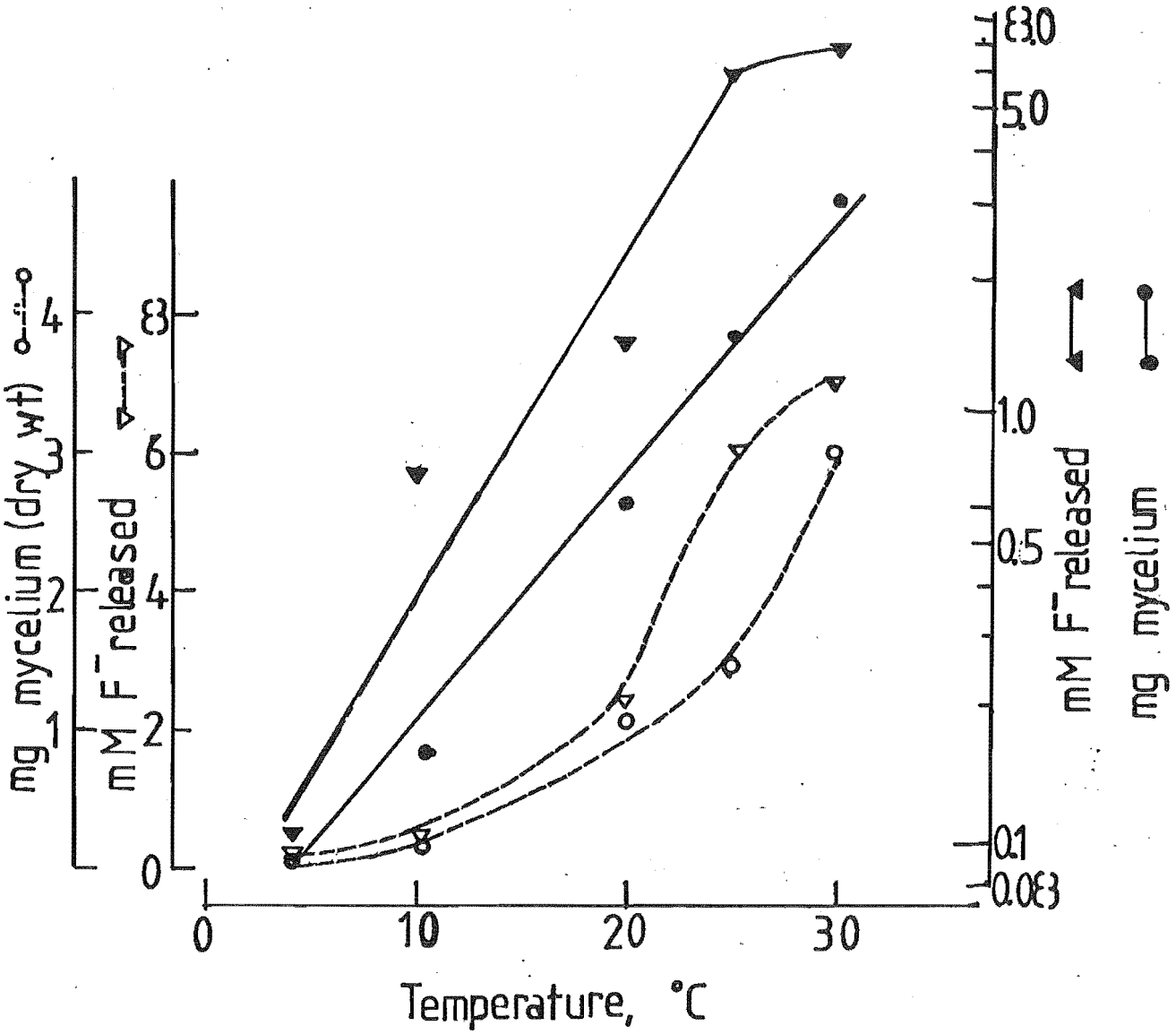


FIG 3.2: EFFECT OF TEMPERATURE ON GROWTH OF *F. solani* AND NaFa DEFLUORINATION



NaFa medium (pH 5.8) was inoculated with spores, incubated at different temperatures for 5 days. F^- released and mycelial yield were determined. Results are averages of triplicates.

Logarithmically transformed functions of NaFa defluorination and mycelial yield are shown to facilitate comparisons of responses of both NaFa defluorination and mycelial growth to temperature.

Again it was found that the response of growth and defluorination of NaFa to increase in temperature was the same (Figure 3.3). At either extreme of the temperature range, little or no growth was observed at 10°C or 40°C respectively, and F^- released into the medium was not evident. The optimum temperature range for growth was 23 - 30°C, and defluorination of NaFa was narrower, 23 - 25°C. With a shorter period of incubation (5 days) as shown in Figure 3.3, growth and defluorination were both higher at 30°C than at 25°C.

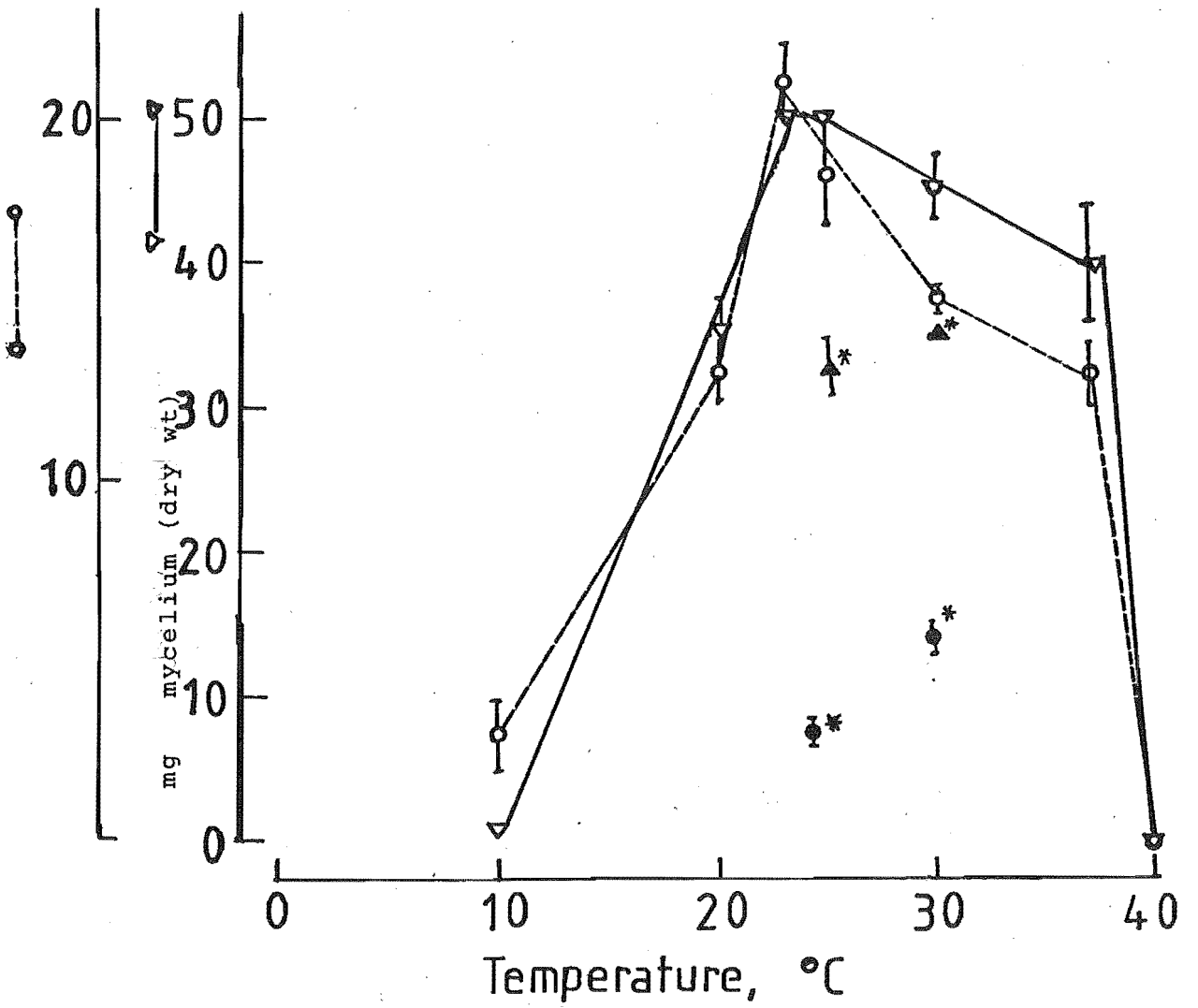
Thus the results show that both growth of *F. solani* and defluorination of NaFa respond to temperature in the same manner, qualitatively and quantitatively.

3.3.5. Effect of Increasing NaFa Concentration

The effect of increasing the NaFa concentration (10-100 mM) on growth of *F. solani* and defluorination of NaFa was investigated in triplicates of 10-ml aliquots inoculated with spores, incubated at 25°C for 5 days. As shown in Figure 3.4 increasing NaFa concentration had no effect on total mycelial yield, averaging 0.9 mg per culture flask. However, the amount of NaFa degraded increased with increasing NaFa concentration from 10 to 20 mM initially applied to the medium. At higher concentration range, the amount of F^- released into the medium was decreased from 7 mM F^- at 20 mM NaFa to 5 mM F^- at 100 mM NaFa.

The experiment was repeated with 9 replicates of 100-ml samples in 250-ml conical flasks. Each flask was inoculated with 3 discs of mycelium of *F. solani* and incubated for 10 days at 25°C. As shown in Figure 3.4 total NaFa degraded showed a small increase with increasing NaFa concentration. The amount of mycelial yield decreased with increasing NaFa concentration.

FIG: 3.3: EFFECT OF TEMPERATURE ON GROWTH OF *F. solani* AND NaFa DEFLUORINATION IN GLUCOSE-AMENDED MEDIUM

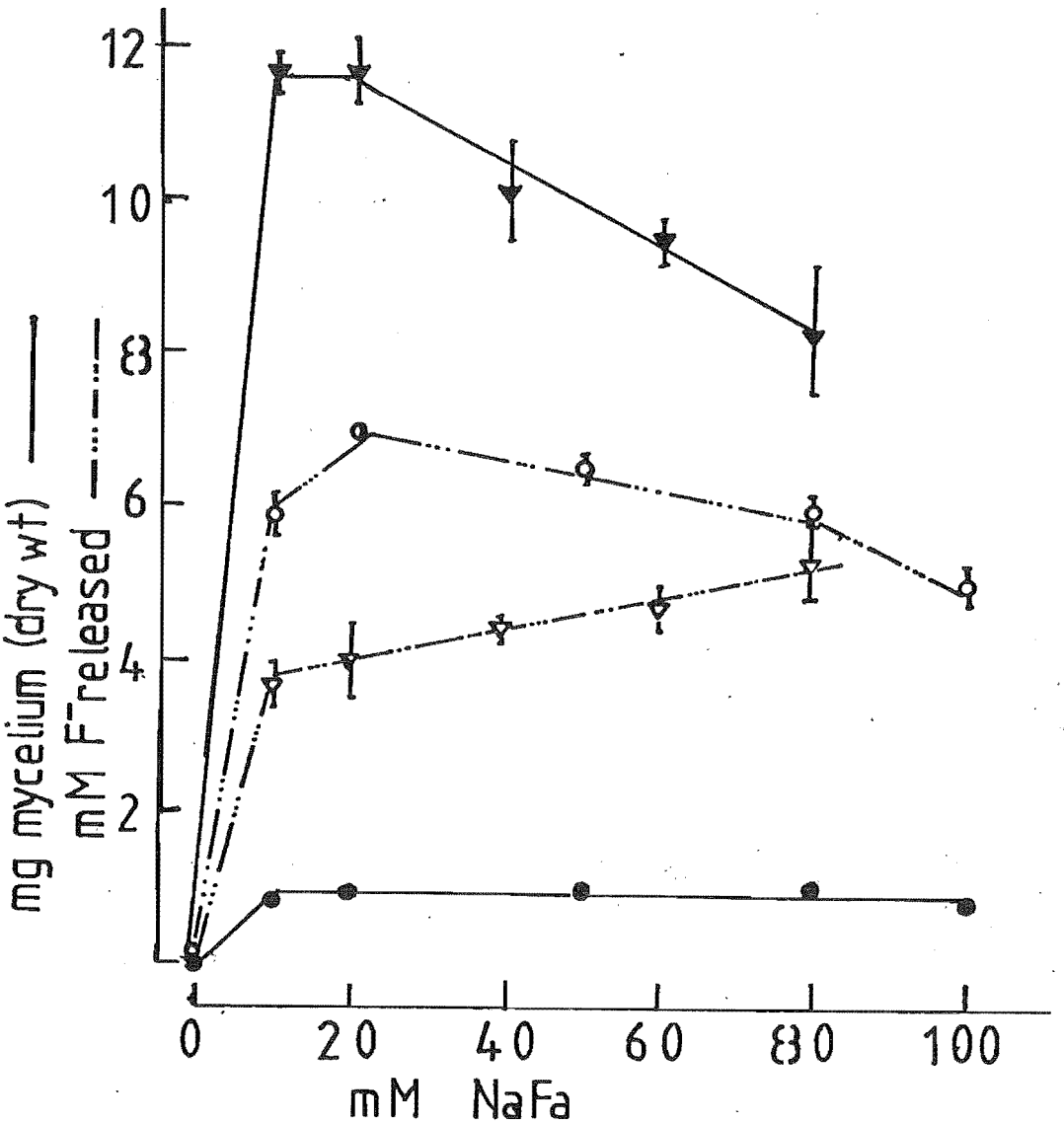


Glucose-supplemented (0.5%, w/v) NaFa medium (pH 5.8) was inoculated with mycelium, incubated at 25°C for 10 days. F^- released and mycelial yield were determined. Results are averages of 10 replicates. Bars represent standard deviation.

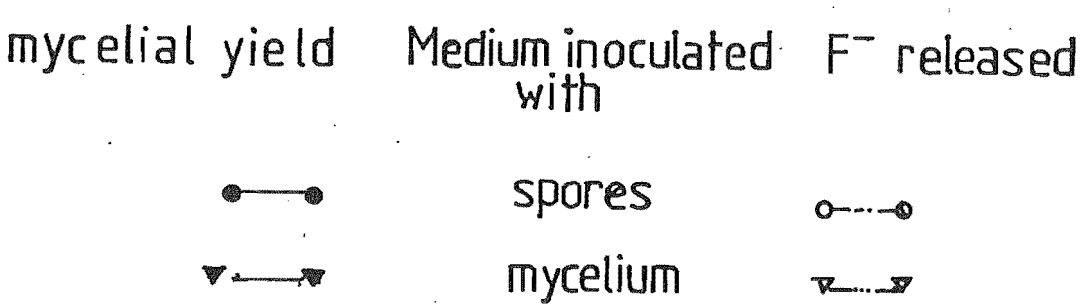
* 5 DAYS' INCUBATION

F^- RELEASED (●) MYCELIAL YIELD (▲)

FIG: 3.4 EFFECT OF INCREASING NaFa CONCENTRATION ON GROWTH OF *F. solani* AND DEFLUORINATION



NaFa medium (pH$_o$ 5.8) inoculated with spores or mycelium, incubated at 25°C for 5 (spore-inoculated) or 10 (mycelium-inoculated) days. F^- released and mycelial yield were determined. Results expressed are averages of triplicates (spore-inoculated) or 9 replicates (mycelium-inoculated). Bars represent standard deviation



3.3.6a. Effect of Carbon Sources

The effect of sodium acetate and glucose on growth and defluorination of NaFa by the five species of fungi was examined in triplicates of 50-ml aliquots of medium containing 10 mM NaFa and 10 mM acetate or glucose, inoculated with spores and incubated at 25°C for 5 days. As shown in Table 3.1, in the presence of acetate, mycelial yield of *F. solani* and *F. oxysporum* was doubled while that of *A. strictum* was tripled. The amount of F^- released into the medium was also increased, by 1.5-fold for both *Fusarium* species and 3-fold for *A. strictum*. Glucose was also found to enhance growth yield of the above 3 species of fungi, by 5- and 10-fold for *Fusarium* species and *A. strictum* respectively, while NaFa defluorination was increased by 2- and 5-fold for *Fusarium* species and *A. strictum* respectively. As shown earlier, *Penicillium* sp. and *Aspergillus* sp. were unable to grow in medium with NaFa as the sole carbon source. In the presence of glucose, significant growth was observed and 5 and 10% of NaFa initially applied to the medium was defluorinated by *Aspergillus* sp. and *Penicillium* respectively. The results thus suggest that fungal defluorination of NaFa could be induced.

The effect of increasing acetate or glucose concentration was investigated using *F. solani*. NaFa concentration was held constant at 20 mM while acetate concentration was varied from 0.0 to 100 mM. At acetate concentrations lower than 5 mM, mycelial yield was similar to that in its absence, averaging 1.2 mg per culture flask. At the higher concentration range, growth was tripled. As shown in Figure 3.5, the response of growth to increasing concentration of acetate was stepwise whereas the amount of NaFa defluorinated varied inversely with increase in acetate concentration (log scale) between 1 and 100 mM. The relationship could be described by the equation

Equation (I) $$[F_a] = [F_o] + b \log [A]^{-1}$$

where [A] = concentration of acetate between 1.0 and 100.0 mM;

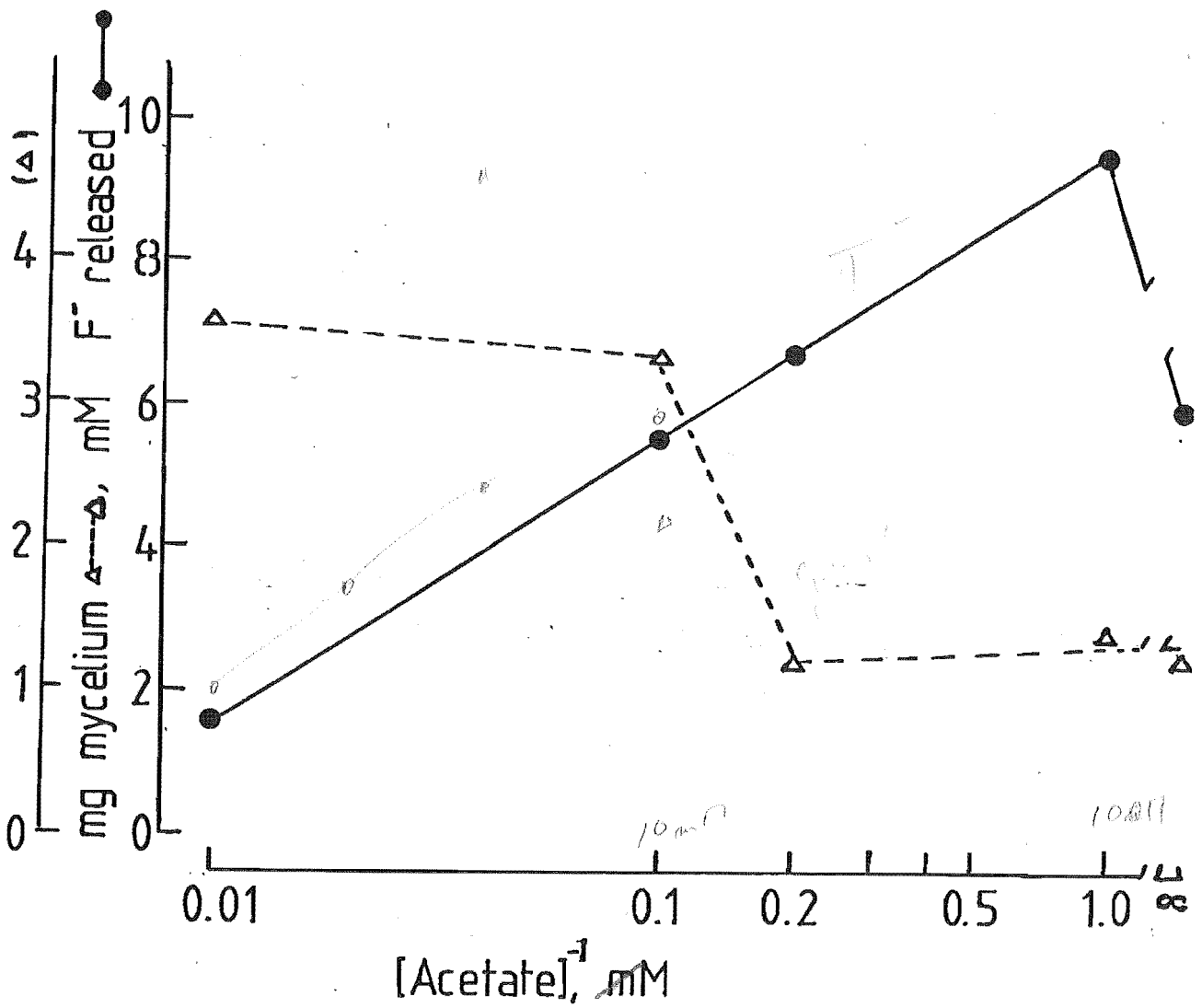
$[F_a]$ = concentration of F^- in the medium;

$[F_o]$ = concentration of F^- at $\log [A]^{-1} = 0$; and

b = slope of the response curve shown in Fig. 3.5.

When computed the equation is $[F_a] = 4 \log 1/[A] - 2.4$, indicating that for every 10-fold the decrease in acetate concentration in NaFa medium, the amount of NaFa degraded increased.

FIG 3.5: CONCENTRATION EFFECT OF ACETATE ON THE GROWTH OF *F. solani* AND NaFa DEFLUORINATION



NaFa media containing different concentrations of acetate were inoculated with fungal spores, incubated at 25°C for 5 days. F^- released and mycelial yield were determined. Results are averages of triplicates.

The effect of increasing the glucose concentration from 0-10 mM was similar to acetate effect in enhancing growth and NaFa defluorination as shown in Figure 3.6. The degree of stimulation of NaFa defluorination decreased with increasing glucose concentration, as observed with increasing acetate concentration. Increment in growth diminished with increasing glucose concentration.

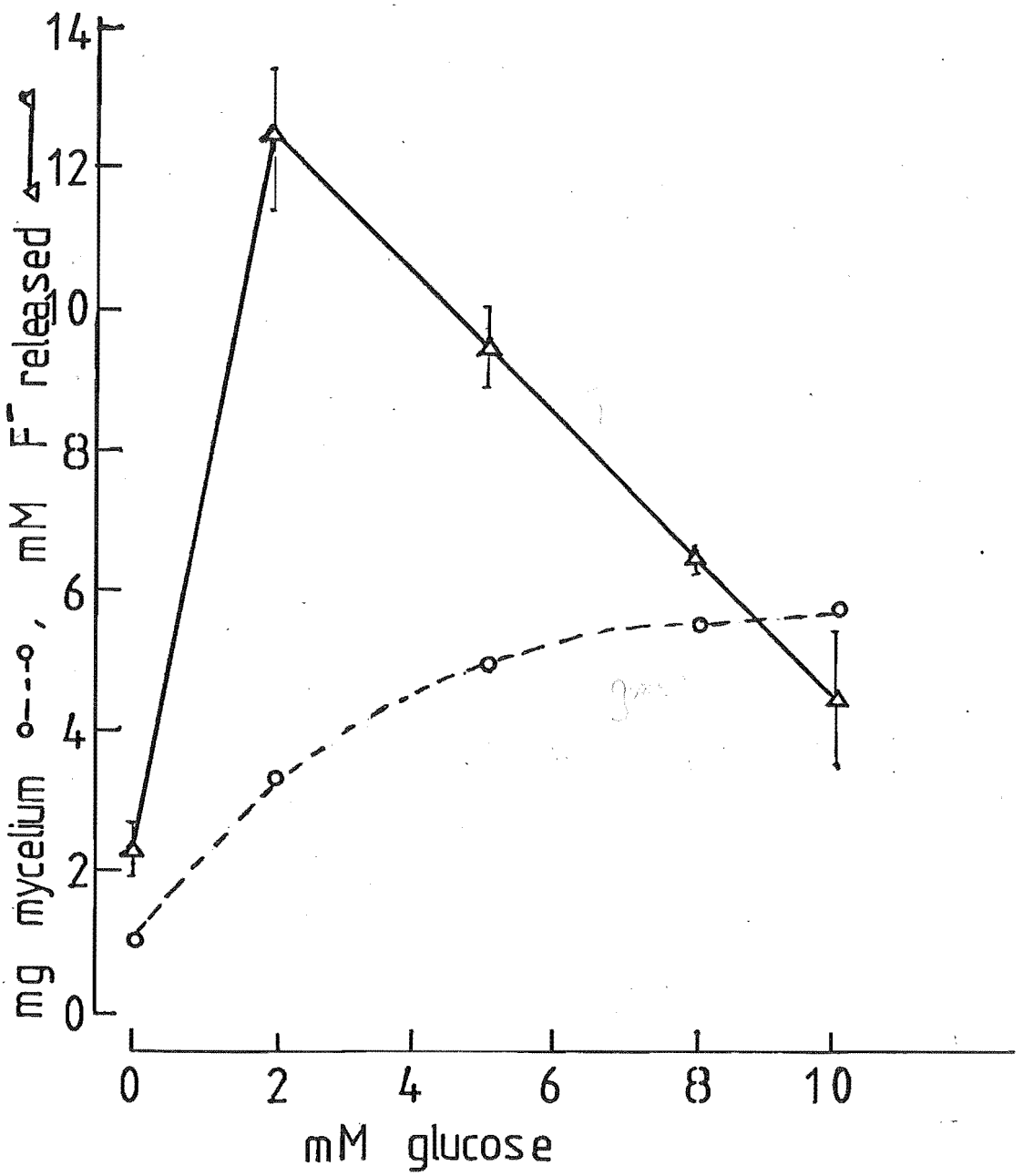
These results thus show that the maximal defluorination of NaFa was influenced by the presence and concentration of acetate or glucose, and was maximal at 1 or 2 mM respectively and decreased with further increase in concentration of either carbon source. Growth was enhanced in the presence of increasing acetate or glucose concentration, but the increment diminished at the concentrations of glucose > 5mM or of acetate > 10 mM.

3.3.6b. Effect of NaFa and glucose

The effect of increasing glucose concentration (0-8 mM) was examined further coupled with variation in NaFa concentration (10-80 mM) in the growth medium. A total of 30 combinations (with 10 replicates of each) of different concentrations of glucose and NaFa was set up. Each replicate, 100 ml aliquot in 250 ml flasks was inoculated with 3 agar discs of mycelium of *F. solani*, and incubated at 25°C for 10 days. As shown in Figure 3.7a with each increase in glucose concentration an increase in mycelial yield was observed at all concentrations of NaFa. But with increase in NaFa concentration mycelial yield was slightly reduced at all concentrations of glucose. Computer analysis of mycelial yields from various combinations of NaFa and glucose showed that there was a positive contribution of the presence of both glucose and NaFa to the growth (Appendix Ia) of *F. solani*, the contribution of glucose being 9 times higher than that of NaFa as deduced from the F_s values.

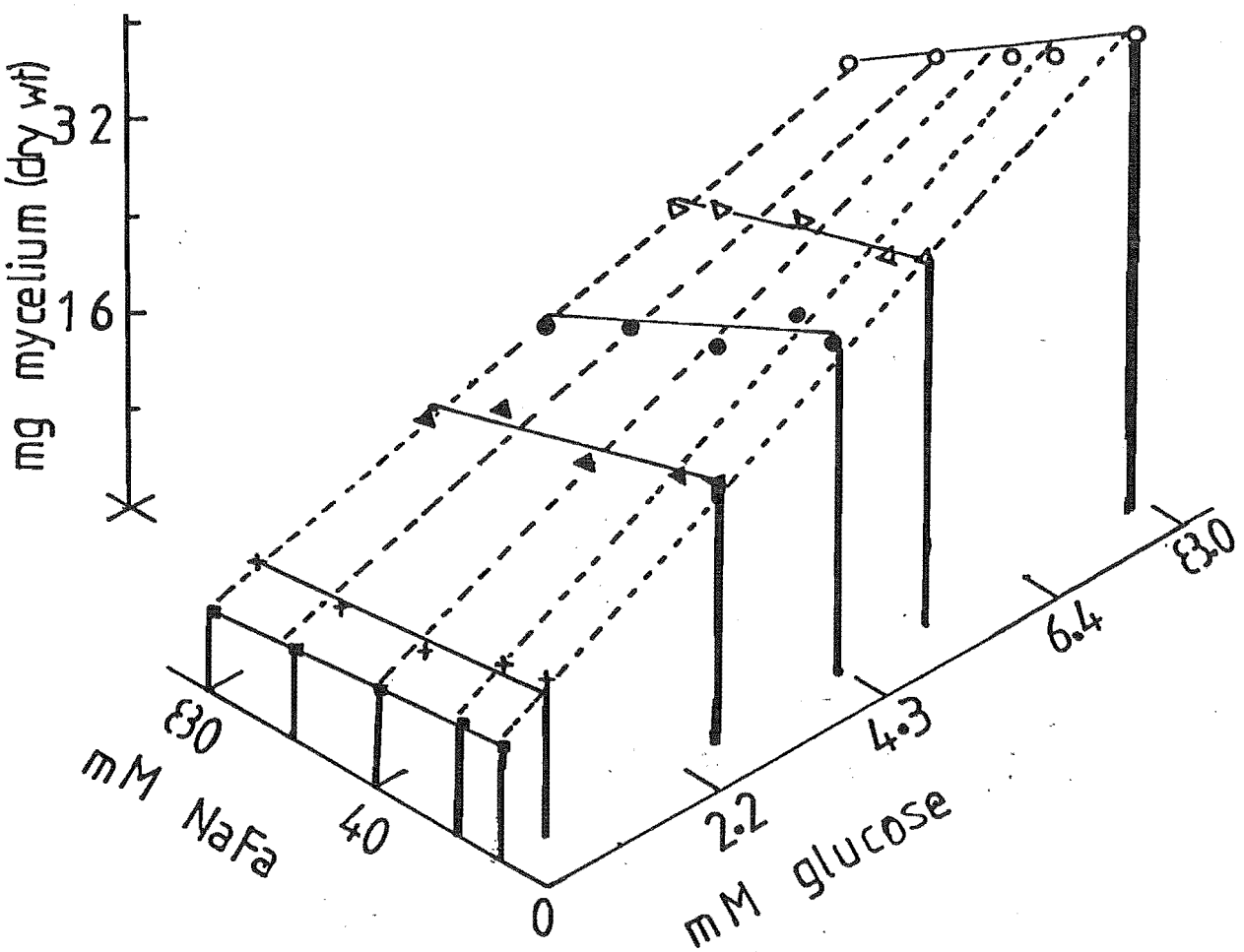
Figure 3.7b shows the effect on defluorination of NaFa. As found earlier, the amount of NaFa defluorinated at each concentration of NaFa increased with increasing glucose concentration up to 2.7 mM glucose for NaFa concentrations between 10 and 60 mM NaFa, and up to 4.3 mM glucose for NaFa level at 80 mM. However with further increase in glucose concentration up to 8.0 mM, the amount of NaFa degraded decreased. At each

FIG: 3.6: CONCENTRATION EFFECT OF GLUCOSE ON THE GROWTH OF *F. solani* AND NaFa DEFLUORINATION



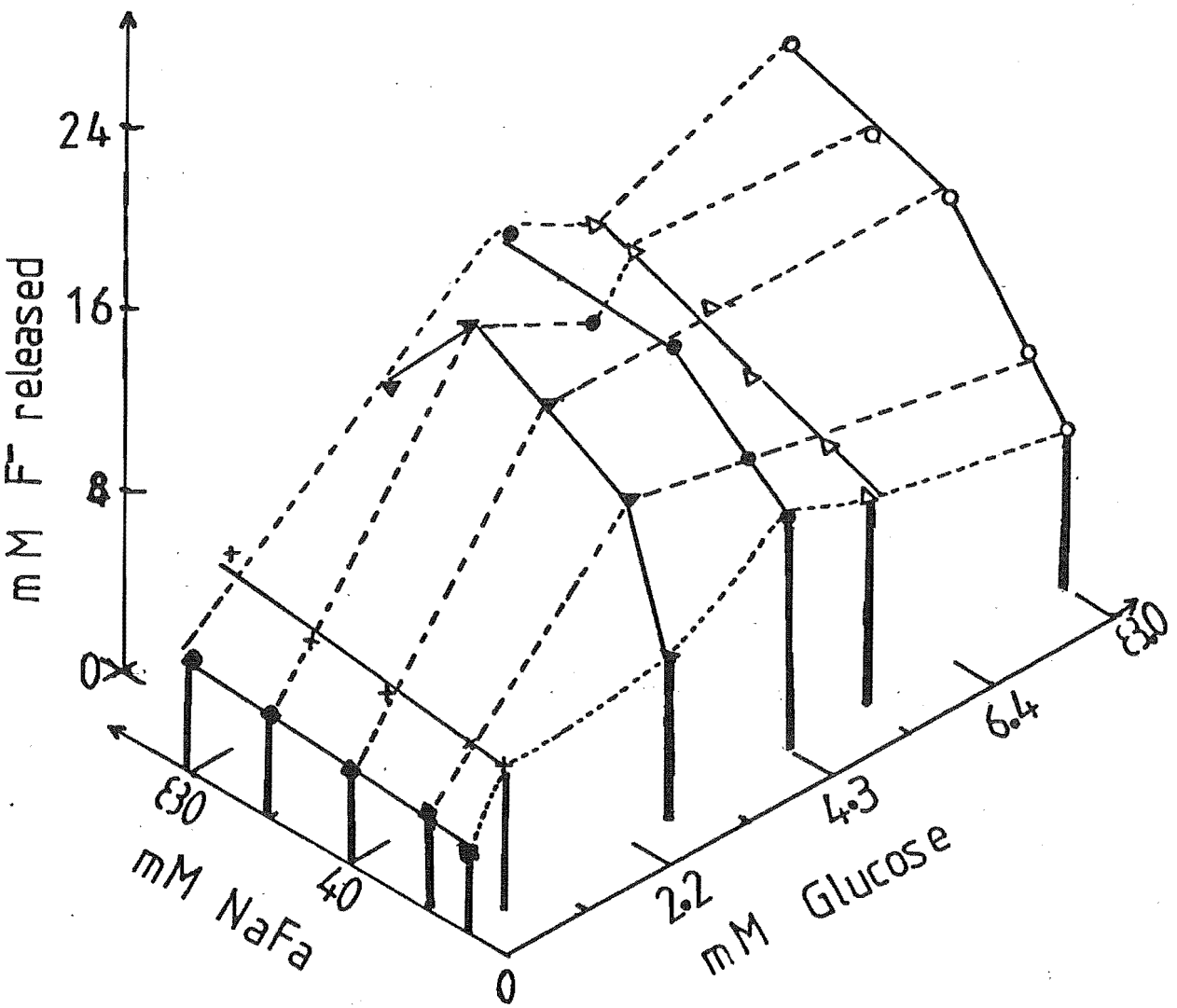
Culture conditions were as described in Fig. 3.5 Results are averages of triplicates.

FIG. 3.7 GROWTH OF *F. solani* AS AFFECTED BY DIFFERENT CONCENTRATIONS OF NaFa AND GLUCOSE



Media containing different concentrations of NaFa and glucose were inoculated with mycelium, incubated at 25°C for 10 days and mycelial yield determined.
Results are averages of 10 replicates.

FIG. 3.7B DEFLUORINATION OF NaFa BY *F. solani* AS INFLUENCED BY DIFFERENT CONCENTRATIONS OF NaFa AND GLUCOSE



Experimental details were as described in Fig. 3.7a, F^- released was determined.
Results expressed are averages of 10 replicates.

glucose concentration (2.7 mM, 4.3 mM and 5.3 mM), the amount of NaFa degraded increased with increasing NaFa concentration up to 60 mM NaFa, but not at 0.5 mM glucose. A tendency of decrease in NaFa defluorination was observed with NaFa concentration > 60 mM. Computer analysis of variance of the results showed that there was a positive interaction between NaFa and glucose present in the medium, but its contribution was about 20 and 10 times less (based on F_s differences) than the contribution to NaFa defluorination by glucose and NaFa respectively (Appendix Ib).

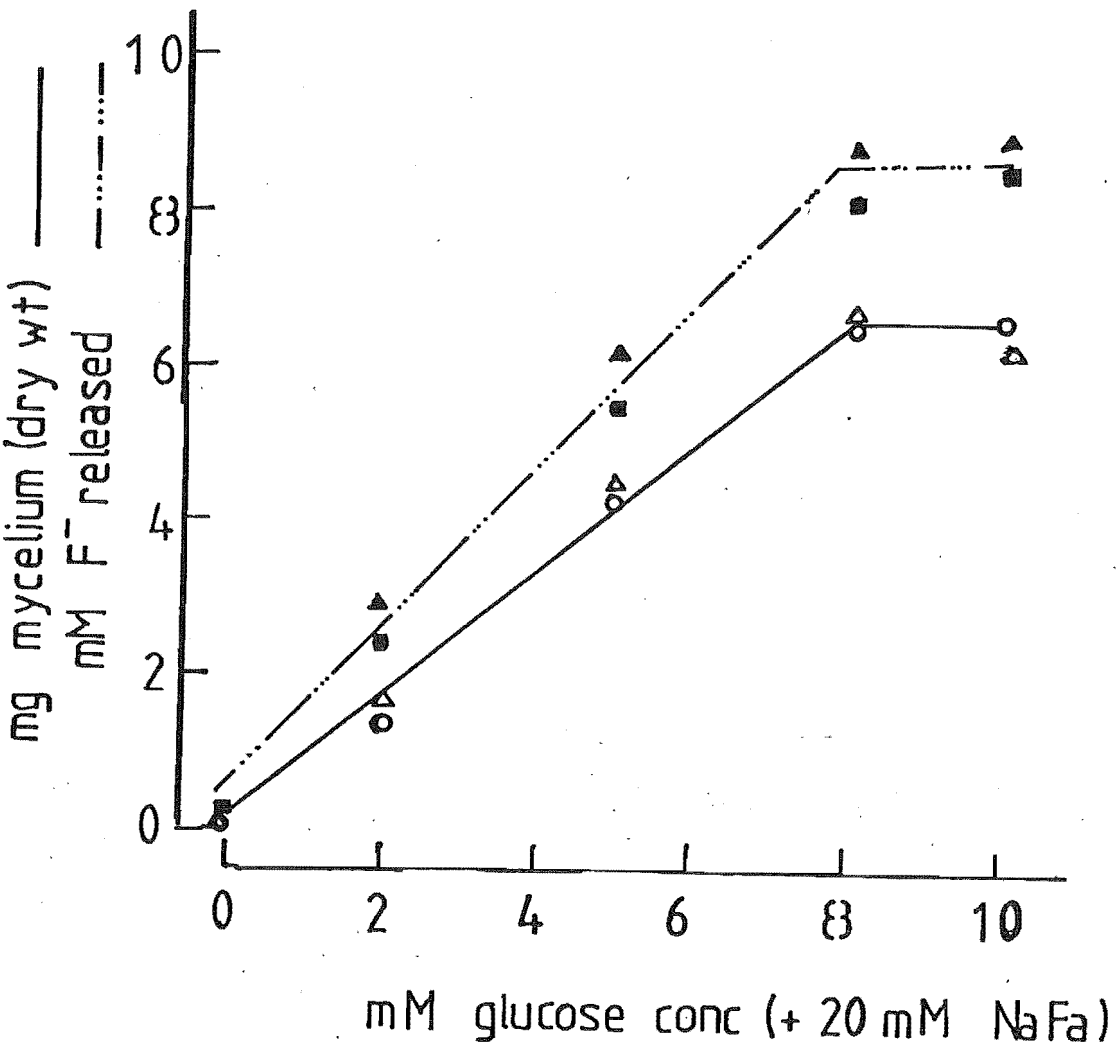
Thus the results show that glucose has a more significant effect on both growth of the fungus and the defluorination of NaFa within the concentration range of each substrate examined.

3.3.7. Effect of Aeration

The effect of aeration or oxygen status of the growth medium was studied by comparing the growth of the fungus and defluorination of NaFa in shake and stationary cultures. In this experiment *Penicillium* sp. was used. As found previously, growth of *Penicillium* sp. in NaFa medium was dependent on the presence of glucose, consequently the effect of aeration on this organism was studied together with the effect of increasing glucose concentration (0-10 mM).

Four replicates of 10-ml aliquots of NaFa (20mM) medium containing various concentration of glucose were inoculated with spores, two were shaken on a rotary shaker (300 rpm) and the rest were held stationary, and incubated at 25°C for 4 days. Total mycelial yield and NaFa defluorinated increased with increasing glucose concentration for both shake and static cultures (Figure 3.8) up to 8 mM. The increment in mycelial yield from shake and static cultures was 0.7± 0.1 mg per mM increase in glucose concentration, and the increment in F^- released into the growth medium was 0.9±0.1 mM for shake cultures and 0.8±0.2 mM. per mM increase in glucose concentration in the growth medium (the slight difference is statistically insignificant at 0.05 probability level). The experiment was repeated using *F. solani* at one concentration of glucose (10 mM) in 20 mM NaFa medium in triplicate, 50-ml aliquots inoculated with spores, and incubated under the same conditions as above. Total mycelial yield in shake and static cultures was 6.3 mg and 6.7 mg per

FIG: 3.8: EFFECT OF AERATION ON GROWTH OF *Penicillium* SP. AND NaFa DEFLUORINATION IN GLUCOSE-AMENDED MEDIUM



NaFa media containing different concentrations of glucose were inoculated with fungal spores, incubated at 25°C for 5 days. F^- released and mycelial yield were determined. Results are averages of duplicates.

mycelial yield Oxygen status of cultures F^- released

shaken

static

replicate respectively whereas a bigger difference was observed in the amount of F^- released into the medium, 2.7 mM in shake culture medium and 3.8 mM in static culture medium, considered to be significant.

In static cultures, the mycelial mats of both *F. solani* and *Penicillium* sp. remained submerged throughout the period of incubation. Toward the end of the incubation period, a surface mat developed and sporulation was observed at higher concentration (8 mM and above) of glucose.

Results thus far show that oxygen status at shake and static level had no effect on both growth and defluorination of NaFa by *Penicillium* sp. The growth of *F. solani* was unaffected but NaFa defluorination was reduced in shake cultures. The presence of glucose did not alter the response of *Penicillium* sp. to aeration.

3.3.8. Rate of Utilization of NaFa

The rate of defluorination of NaFa and growth of *F. solani* was followed for a period of 10 days. Triplicates of 50-ml medium with NaFa (20 mM) as the sole carbon source were inoculated with spores and incubated at 25°C, without shaking. Growth was not visible in day 1, but from day 2 onward, growth was linear as shown in Figure 3.9a. A very short period of exponential growth would be expected between day 1 and 2. The linear phase of growth was fitted to the equation to obtain the rate of growth:

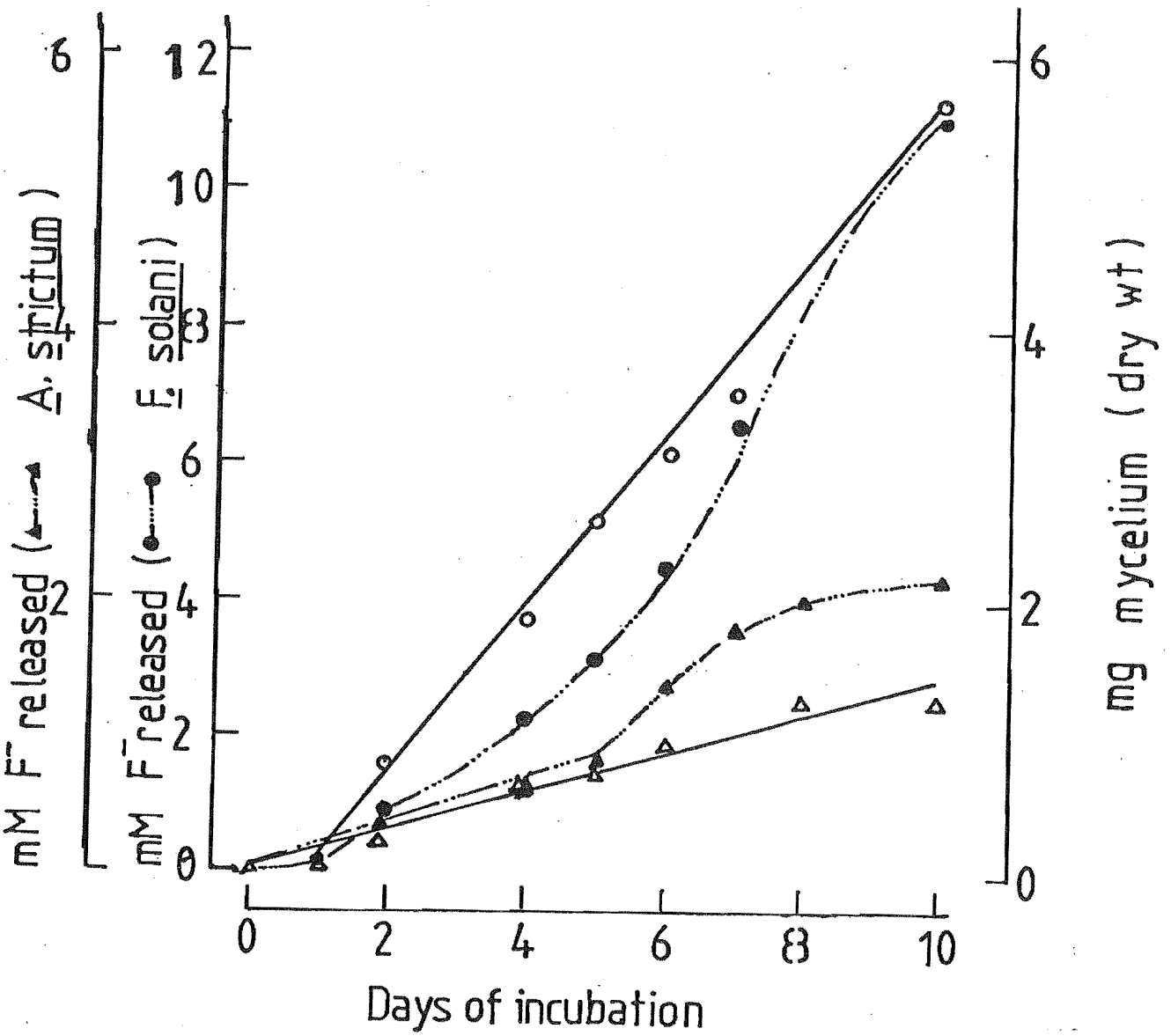
Equation (II) $M = M_o + bT$ where $2 \leq T \leq 10$ days

M = dry weight of mycelial yield at time T (days of incubation);
M_o = mycelial yield at T = 0. This value is hypothetical only because the equation is true for $2 \leq T \leq 10$ days; and
b = rate of growth

The rate of growth and the accompanying statistics were computed using a programmable calculator and are given in Figure 3.9a.

Unlike the growth curve, defluorination of NaFa was non-linear with respect to time, showing an initial lag period as the common feature. Defluorination of NaFa resembled a power function (Figure 3.9a).

FIG. 3.9A RATES OF GROWTH AND NaFa DEFLUORINATION BY *F. solani* AND *A. strictum*



Medium with NaFa as the sole carbon source was inoculated with fungal spores and incubated at 25°C. F^- released and mycelial yield were determined at intervals. Results are averages of triplicates. (note dual ordinates of F^- released).

rate of mycelial yield	Fungal species	F^- released
0.54 ± 0.003 mg/day	*F. solani*	
0.14 ± 0.002 mg/day	*A. strictum*	

Equation (IIIa) $[F] = [F_o] \; T^{b'}$ where $2 \leq T \leq 10$ days

Equation (IIIa) could be linearly regressed to give the equation (IIIb):

Equation (IIIb) $\log [F] = \log [F_o] + b'' \log T$ where $2 \leq T \leq 10$ days

$[F]$ = concentration of F^- released in culture medium at time T (days of incubation);

$[F_o]$ = concentration of F^- at log T = 0; and

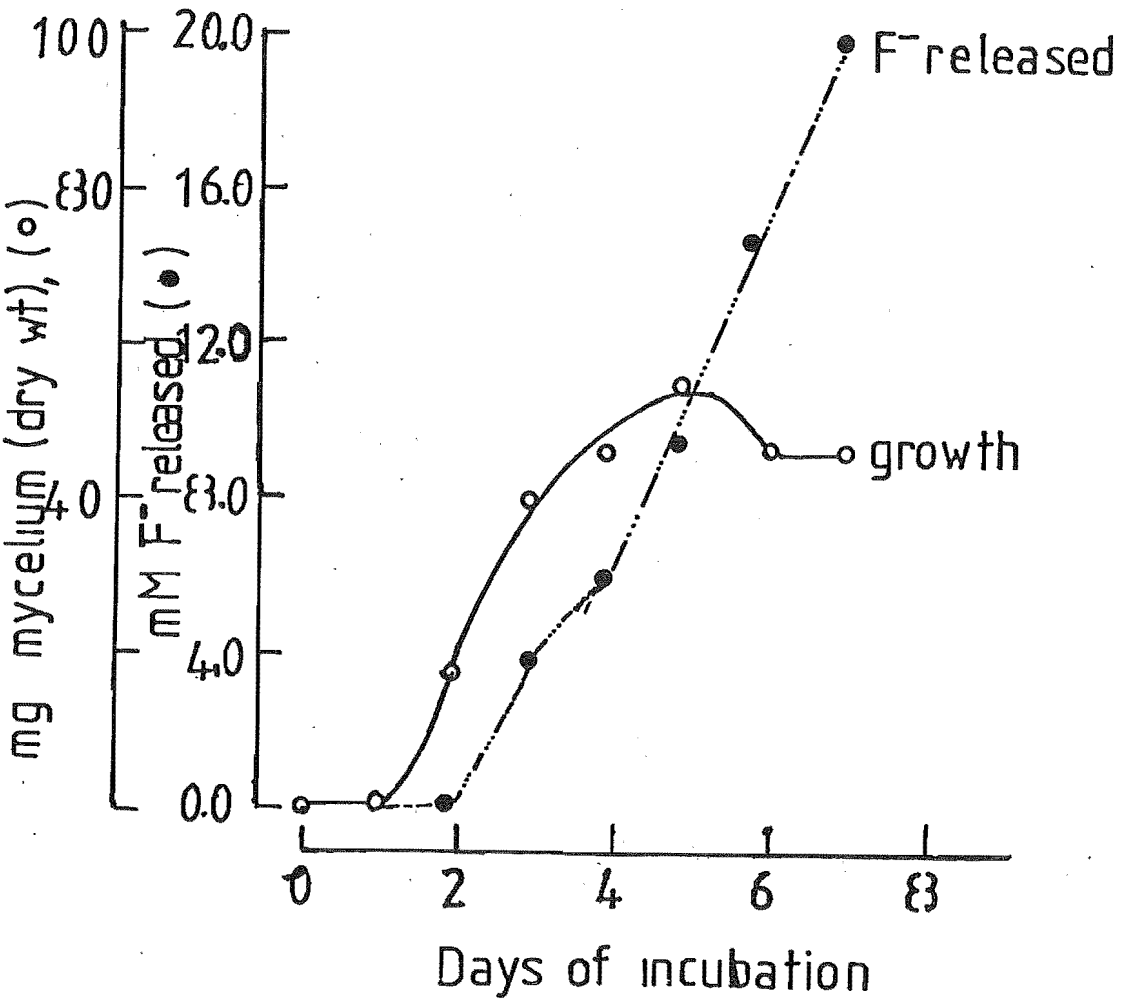
b" = rate of defluorination of NaFa (ln b')

b" was computed using logarithmically transformed data, and other statistics are given in Figure 3.9c.

The experiment was repeated using *A. strictum*. Growth of *A. strictum* was more or less linear after an initial lag and possible brief exponential phase. Growth reached the stationary phase at day 8 as shown in Figure 3.9a. During the linear phase of growth, the rate of increase in mycelial yield of *A. strictum* was 4 times slower than that of *F. solani* as shown in Figure 3.9a. Defluorination of NaFa by *A. strictum* could be represented by the same power function as described above and shown in Figure 3.9c. The relative rate of defluorination of *A. strictum* was found to be 0.76 times that of *F. solani* (Figure 3.9c).

The effect of 2.8 mM glucose on the rate of growth and defluorination of NaFa by *F. solani* was also followed. Ten replicates of 150-ml medium in 1-litre Roux bottles were inoculated with 5 agar discs of mycelium and incubated for 7 days at 25°C. Growth of *F. solani* in glucose supplemented medium showed four qualitative phases: an initial lag (1 day) followed by an accelerating phase, a decelerating period and declining or stationary phase, as shown in Figure 3.9b. During the exponential phase (days 1 to 3), mycelial yield doubled each day. Between day 3 and day 5, growth decelerated and became linear. There was a sharp decline in mycelial yield at day 6 and thereafter became constant. While growth underwent these various phases, defluorination of NaFa proceeded at an accelerating rate (as described by a power function) after a two-day lag period, as depicted in Figure 3.9b and Figure 3.9c. The relative rate of defluorination of NaFa in the presence of glucose was found to be 1.5 times that in its absence (from spore-

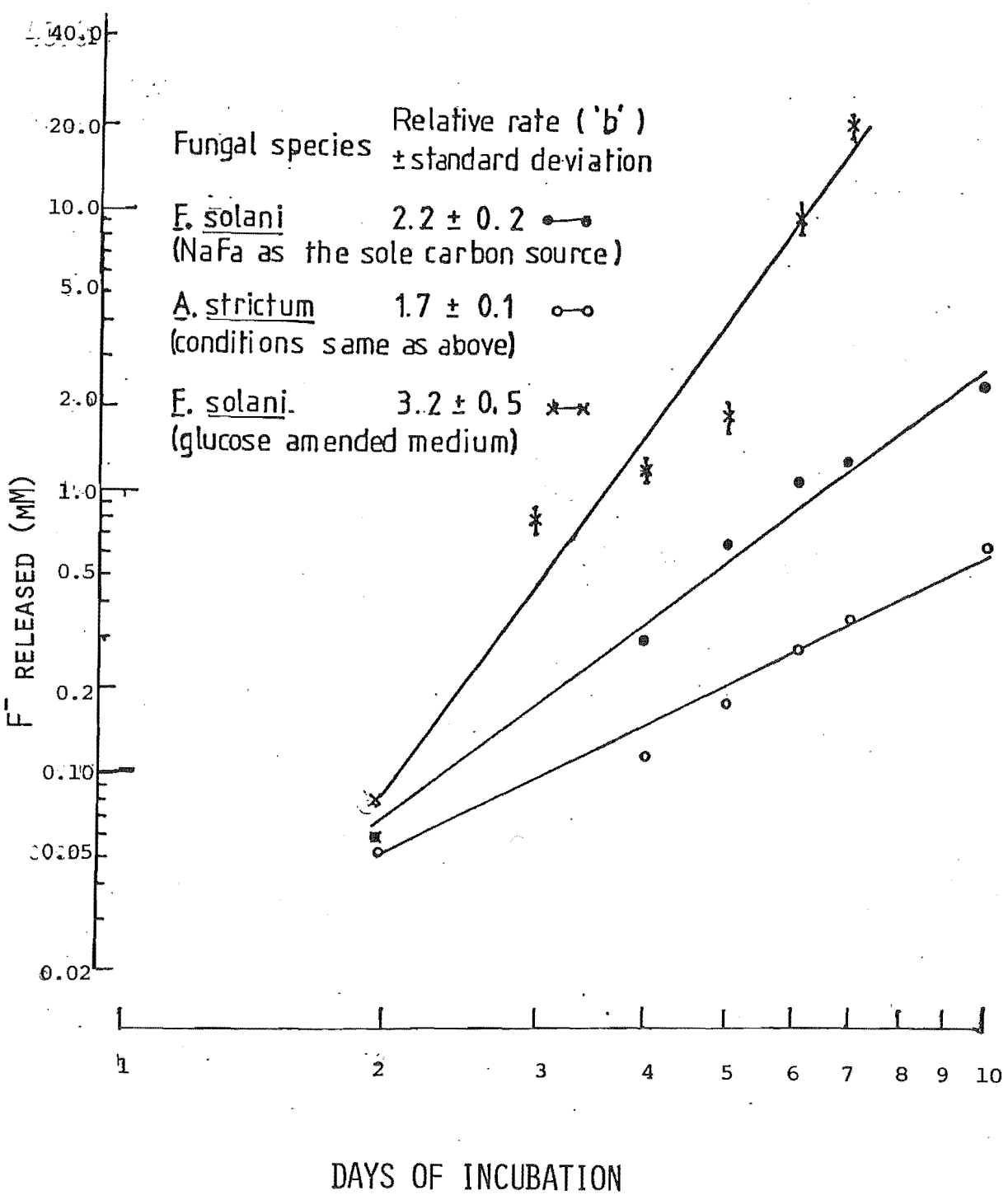
FIG 3.9B RATE OF GROWTH OF *F. solani* AND NaFa DEFLUORINATION IN GLUCOSE-AMENDED MEDIUM



NaFa medium supplemented with 2.8 mM glucose was inoculated with mycelium, incubated at 25°C. F^- released and mycelial yield were determined at intervals. Results expressed are means of 10 replicates.

Phase of growth	duration
Lag	day 0–1
Accelerating	days 1–3
Decelerating	days 3–5
Declining to stationary	days 5–7

FIG 3.9c: TRANSFORMED FUNCTIONS TO DETERMINE AND COMPARE RELATIVE RATES OF DEFLUORINATION BY FUNGI



inoculated cultures of *F. solani*) and twice that of *A. strictum*.

Results thus show that defluorination of NaFa was independent of the state of growth of fungus. In the presence of glucose, mycelium inoculated cultures defluorinated NaFa at a higher rate than in its absence with spore-inoculated cultures of *F. solani*. *F. solani* was more active both in growth and defluorination of NaFa than *A. strictum*.

3.3.9 Localisation of NaFa Defluorination by *F. solani*

The following experiment was conducted to try to find whether NaFa defluorination was intra- or extracellular. Four of the 5 triplicates of 100-ml aliquots of 20 mM NaFa medium were inoculated with spores of *F. solani* and incubated for 12 days at 25°C. The uninoculated triplicate served as control. At day 5, one inoculated triplicate was removed and concentration of F^- in each culture flask was measured, showing that 16% of NaFa initially present had been defluorinated (Table 3.3). Mycelium in this triplicate was then killed by autoclaving at 120°C for 30 mins, and returned to the incubator. At day 8, mycelium from another triplicate was removed, the culture filtrate filter-sterilised, and returned for further incubation. F^- concentration in another triplicate was measured, and the mycelium killed and returned for further incubation as before.

Table 3.3 Localisation of NaFa defluorination

Incubation period (Days)	F^- concentration (mM) in culture flasks			
	Uninoculated Control	Dead mycelium	Culture filtrate	Living mycelium
0	0.06			
5: (mycelium killed)	0.06	3.2		
8: (mycelium killed)			8.0	
12	0.06	3.2	8.0	20.0

The experiment was terminated at day 12. Results presented in Table 3.3 show that in culture flasks with dead mycelium there was no further increase in F^- concentration after further 3 and 7 days incubation, suggesting that NaFa defluorination was

not merely a physically catalysed reaction on the surface of mycelium. The culture flasks from which mycelium was removed showed no further increase in F^- content, suggesting that the enzyme may be intracellular, or, if extracellular, was unstable. Increase in F^- content was observed only in those culture flasks with living mycelium, suggesting that defluorination of NaFa was enzyme-catalysed.

The possibility that the enzyme may be intracellular was investigated further. Mycelium harvested from 10-day old cultures of *F. solani* grown in 20 mM NaFa medium was washed several times in phosphate buffer (pH 6.5), then ground with sand in 15 ml phosphate buffer. The mycelial homogenate was centrifuged at 1,000 g for 10 mins. The precipitate of cell debris was re-suspended in buffer and the supernatant was centrifuged further at 25,000 g for 30 mins. Supernatant and precipitates were assayed for defluorinating activity in a reaction mixture consisting of 1.5 ml of the above preparation and 1.5 ml 20 mM NaFa, incubated for 2 hours at 25°C. Yields of both F^- and glycolate were measured in triplicates.

The activity of the various fractions is shown in Table 3.4. Maximum defluorinating activity was detected in the supernatant fraction and some activity was detected from the fraction precipitated at 25,000 g indicating that defluorinating enzyme resided in the cytoplasm. The results thus confirm that defluorination of NaFa was not a physical process.

Table 3.4 Defluorinating activity of Various Fractions of Mycelial Homogenate

Reaction Mixture Fraction + 10 mM NaFa	Breakdown Products of NaFa	
	Inorganic F^- (mM) released	Glycolate (mM)
Sand (control)	0.0	0.0
Sand and mycelial debris	0.0	0.0
Precipitate (1,000 g)	0.0	0.0
Precipitate (25,000 g)	0.21	0.17
Supernatant (25,000 g)	0.50	0.40
Boiled supernatant	0.0	0.0
Culture filtrate	6.0	0.0
Boiled filtrate	6.0	0.0

Reaction mixture incubated for 2 hours at 25°C. Results are averages of triplicates. Culture filtrate was the medium in which *F. solani* had grown and the mycelium removed.

Table 3.5: Defluorinating Activity of Bacterial Cells Previously Adapted to Different Carbon Sources

Growth Substrate (20 mM)	Defluorinating Activity (F^- released, mM) NaFa (10 mm)	Fluoroacetamide (10 mM)
NaFa	2.3	1.6
Fluoroacetamide	1.9	1.8
Chloroacetate	1.8	0.5
Glycolate	0.5	0.4
Succinate	0.3	0.3

Reaction mixture (cell suspensions + 10 mM NaFA or fluoroacetamide) incubated at 25°C for 1 hour. Results are averages of triplicates.

3.3.10 Nature of Synthesis of Defluorinating Enzyme

The nature of synthesis (inductive vs constitutive) of the defluorinating enzyme was investigated using a *Pseudomonas* sp isolated previously. The bacteria were adapted to grow in mineral salts media differing only in the carbon source supplied (20 mM) - NaFa, fluoroacetamide, chloroacetate, glycolate or succinate. Inoculum was obtained from bacteria previously grown in 20 mM NaFa medium. Bacteria were cultured in 500-ml conical flasks containing 250 ml growth medium, shaken at 300 rpm on rotary shaker for 2 days at 25°C. Bacterial cells were harvested by centrifuging at 5,000 g for 20 mins, washed several times in phosphate buffer and suspended in the same buffer, then activity assayed as before except that incubation was for 60 mins. Equal concentrations of cell suspensions were used, 8.6 mg (dry wt) per replicate. Results were given in Table 3.5. Bacterial cells previously adapted to NaFa or chloroacetate were able to defluorinate NaFa to similar extent, but not fluoroacetatamide. Glycolate- or succinate-adapted cells showed some defluorinating activity and that could be either constitutive or, more probably, due to limited adaption during the 60 min incubation (Stanier, 1947). It may be deduced that the synthesis of defluorinating enzyme is inductive.

3.4 DISCUSSION

3.4.0. Environmental factors such as nutrients, pH, temperature and aeration influence the growth of organisms and their metabolic processes. Each of these factors was investigated independently because a continuous-culture apparatus or chemostat, convenient for the manipulation of physical and chemical parameters, was not available. The effect of these factors on the growth (mycelial yield) of the organism and on NaFa defluorination (F^- released into the medium) was determined.

3.4.1. Determination of Effect on Growth and NaFa Defluorination

In the present studies growth of fungi was determined by measuring the dry weight of mycelium produced in submerged cultures. Other parameters of growth commonly used include turbidimetric measurements, radial colony extension on solid agar medium, and less frequently, nitrogen determination. Turbidimetric measurement was unsuitable for present studies since the fungi used were filamentous. Radial extension of fungal colony on a solid medium was considered inadequate for present studies for several reasons. Preliminary experiments showed that some fungi (*Aspergillus* sp. and *Penicillium* sp.) which were able to grow on solid NaFa medium were unable to grow in liquid medium of similar chemical composition. Furthermore unnecessary complication in procedural extraction of inorganic F^-, essential for the determination of defluorination, would be introduced by the use of solid media. The inadequacy of colony growth on agar plates for studying the effect of nutrient status and for comparison among different species of fungi has been demonstrated in a comprehensive study by Trinci (1969; 1971). The peripheral growth zone of hyphae varies in width and septation depending on species of the fungi and nutrient status of the medium, therefore radial colony extension on agar medium is not a meaningful growth parameter. Measurement of growth by determination of cellular nitrogen was not used although the method would give a better estimation of growth or protoplasm increase. Because the present work was equally concerned with the breakdown and assimilation of NaFa, nitrogen determination may exclude products such as fats or lipids, formed from oxidative assimilation of NaFa.

In the present studies on the effect of an environmental

variable on growth and defluorination of NaFa, the total yield rather than relative rate of growth or defluorination (except for kinetic studies) was used as a parameter for comparison. Relative rate of mycelial yield or F^- release would be more meaningful than total yield because the use of the latter parameter entails the assumption that at the time of measurement the organism in all the various treatments was at the same phase of growth or defluorination-lag, exponential or stationary. This assumption could either over - or underestimate the response of the organism to an environmental variable, especially in growth response because the phases of growth kinetics are relatively more varied and distinct than those of defluorination. The assumption would not affect the data on the effect of carbon or nitrogen sources to the same extent as data on effect of aeration and temperature.

In all studies, NaFa defluorination was determined by measuring F^- released into the medium. Consequently the accuracy of estimating NaFa degradation is influenced by the rate at which F^- is released. It is assumed that the organisms do not differ in their F^--binding properties and kinetic studies showed a complete recovery of F^- from culture medium when known amounts of NaFa supplied to the growth medium were completely defluorinated.

3.4.2. Effect of Carbon and Nitrogen Source, Temperature, Aeration and pH

The ability of five species of soil-fungi, *Fusarium solani* (Mart.) Sacc., *F. oxysporum* (Schlecht), *Acremonium strictum* W.Gams, *Penicillium* sp., and *Aspergillus* sp. to defluorinate NaFa and use it as the sole carbon source for growth was examined. The results showed that *Fusarium* species were relatively more active in NaFa deflourination and growth compared to *A. strictum*, while *Penicillium* sp. and *Aspergillus* failed to defluorinate NaFa and grow in a medium with NaFa as the sole carbon source and ammonium-N as the sole nitrogen source. Further studies on the effect of nitrogen sources (NH_4^+, NO_3^- and urea) showed that maximal growth and NaFa-defluorination by *Fusarium*

species occurred in NH_4^+ medium whilst *A. strictum* was equally active in either NO_3^- or NH_4^+ medium, indicating that the influence of nitrogen sources was more significant on *F. solani* than on *A. strictum*. Poorer growth of *F. solani* and *F. oxysporum* in NO_3^- medium may be attributed to an increased demand on metal and reductant supplies for reducing NO_3^- to NH_4^+ for assimilation. The reduction of NO_3^- requires such cofactors as FAD, Fe^{++}, Mo^{6+}, Cu^{++}, Mg^{++} and Mn^{++} (Pateman & Kinghorn, 1976). Except for Fe^{++} and Mg^{++}, the other metals were not supplied to the growth medium but were probably present as trace impurities so that significant growth and defluorination of NaFa were still observed. In urea medium, *F. solani* showed poorer growth and defluorinating activity despite the presence of higher (twice) total N concentration than in NH_4^+ medium. Growth in urea medium indicates that *F. solani* could make use of urea to some extent with a lowered capacity to defluorinate NaFa. It is possible that urea may compete with NaFa as carbon source for growth. NaFa defluorination was found to be stimulated at low ($\leq$ 40 mg NH_4^+ l^{-1}) and inhibited at higher concentration ($\geq$ 40 mg NH_4^+ l^{-1}) of ammonium-N.

In the presence of more easily assimilated carbon sources such as acetate, NaFa defluorination and growth of both *Fusarium* species and *A. strictum* were enhanced. Greater enhancement was observed with equal molar concentration of glucose present in NaFa medium. This would be expected because on a molar basis, glucose contains 3 times as much carbon as acetate. *Penicillium* and *Aspergillus* sp. which failed to grow in medium with NaFa as the sole carbon source, defluorinated NaFa and grew in glucose-supplemented NaFa medium. This indicates that the potential to defluorinate NaFa exists in these fungi and that the type of carbon source plays a critical role in the expression of this potential. Glucose or acetate could supply the energy and intermediate needed by these fungi to manifest the potential for NaFa defluorination.

The nature of enhancement was studied further by varying the concentration of acetate or glucose supplied to NaFa medium. The degree of enhancement effect of acetate on defluorination was found to be inversely proportional to the concentration of acetate supplied, resembling a substrate-competition situation. This type of competition is conceivable because fluoroacetate is

an analogue of acetate. Competition could reside at the site of entry into the cell, since the site of NaFa defluorination was found to occur within the mycelium. If acetate is actively transported through the cell membrane by a carrier molecule, it is possible that this carrier molecule may lack specificity and fail to distinguish acetate from fluoroacetate. Such a lack of specificity is common; for example: the cellular uptake of urea (Cochrane, 1958; Pateman & Kinghorn, 1976), and the failure of aconitase to differentiate citrate from fluorocitrate (Peters, 1957). Another site of competition may lie in the metabolic pathway which will be elaborated in Chapter 3: 3.4.4. The effect of increasing glucose concentration in NaFa medium was similar to the acetate effect on NaFa defluorination by *F. solani* - a concentration-dependent effect. Although glucose is not an analogue of NaFa, a competitive type of inhibition could still result from its metabolites as discussed in Chapter 3.4.4. Whilst defluorination of NaFa by *F. solani* varied inversely to increasing glucose concentration, defluorination of NaFa by *Penicillium* sp. increased with increasing glucose concentration. A further difference was observed in the response of *F. solani* and *Penicillium* sp. to increased oxygen status of the growth medium: NaFa-defluorination by *F. solani* was lower in medium with higher oxygen tension whereas *Penicillium* sp. was unaffected. An attempt to explain the difference is presented in Chapter 3.4.4.

Studies on the nutritional composition of the growth medium showed that the ability of fungi to defluorinate was, to some extent, influenced by the type and concentration of nitrogen or carbon sources available. Thus the inability of a particular organism to act on any compound in a given medium or set of circumstances does not necessarily indicate that the potential to do so is absent. Studies with the bacterium, *Pseudomonas* sp. showed that the ability to degrade NaFa was expressed in cells previously grown on NaFa or its analogues but not very evident in cells previously grown in other compounds such as glycolate or succinate (Table 3.5.)

Besides nutrients, pH has also been shown to exert a great influence on growth and defluorination of NaFa by *F. solani* and *A. strictum*. Growth of *F. solani* was optimal

within the pH range, 5.8-7.2 while NaFa defluorination was relatively more sensitive to pH with maximum observed at pH 5.8. The defluorination of NaFa by *A. strictum* was relatively less sensitive to external pH while its growth was maximal at pH 5.8. The differential sensitivity of *A. strictum* and *F. solani* in defluorination of NaFa to pH may imply different permeability properties of their cell membranes. The mechanisms by which pH affect the growth and NaFa defluorination will be discussed in Chapter 4: 4.4.5.

Economic coefficients (3.3.2) with NaFa as the sole carbon source was shown to be lowest for both *F. solani* and *A. strictum* at pH 7.8, and highest at 5.0 and 6.5 for *F. solani* and *A. strictum* respectively. The pH at which the highest economic coefficient or utilization of NaFa was observed did not coincide with the pH for the maximal defluorination of NaFa by *F. solani*, thus indicating that under conditions sub-optimal for growth, NaFa defluorination could still proceed. It appears that defluorination of NaFa is to some degree, independent of the state of growth of the fungus as deduced from the above and the difference in sensitivity of growth and defluorination of NaFa by *A. strictum* and confirmed later in kinetic studies (3.4.3.).

This limited degree of independence was also observed from the effect of temperature. Within the temperature range, 4-25^{o}C growth and defluorination of NaFa showed similar qualitative and quantitative response to temperature. With further increase from 25^{o}C to 30^{o}C, growth of *F. solani* doubled whilst defluorination of NaFa increased by only 40%. This is not uncommon because temperature affects different enzyme systems differently. A given metabolic process such as the synthesis of penicillin by *Penicillium chrysogenum* is maximum at 20^{o}C while maximal growth occurred at 30^{o}C (Deverall, 1965).

Growth and defluorination of NaFa has been shown to occur with varying degrees of success over fairly wide pH range (pH 5.0-7.8) and temperature range (10^{o}C < temperature < 40^{o}C); with the lower limit at pH 5.0 and lower limit of temperature 10^{o}C; and upper limit at pH above 7.8 or temperature above 30^{o}C, but less than 40^{o}C. How temperature and pH would interact on growth

and defluorination of NaFa by fungi or other organisms was not investigated. Results obtained thus far indicate that under conditions adverse or favourable to growth, NaFa defluorination proceeds to varying degree depending on the type of organism present. For instance, increasing glucose concentration increased defluorination of NaFa by *Penicillium* sp. but decreased that by *F. solani* whilst the growth of both species of fungi was enchanced. From the studies of the various factors, the carbon substrate appears to exert the greatest influence on growth of the organism, its ability to defluorinate NaFa and the rate of NaFa defluorination. However, the influence of carbon sources is dependent on their availability which is to some extent determined by pH. NaFa defluorination was also influenced by temperature. Of significance is that the ability of an organism to defluorinate NaFa is inducible.

For use as a pesticide, NaFa is usually supplied as NaFa-treated baits such as carrots, bran, pollard, molasses or meat which are high in nutrient content. NaFa is usually adsorbed to carrot baits by immersing the latter in NaFa solution. The internal moisture content may be sufficient to initiate microbial growth on the rich substrate with consequent metabolic and biosynthetic activities. Because of the way by which NaFa baits are processed, handled and applied, an early establishment of a microflora could occur by contamination from human handling of treated baits, from NaFa solution, or during transportation. The high toxicity of NaFa requires specific stations for preparation of NaFa baits or re-packaging of NaFa so that the microflora in these areas would have had ample opportunity to develop defluorinating systems. Thus when baits are supplied or transported the induced microflora would have been carried along to the new destination and consequently the nutrient and microbial diversity or numbers of the areas to which NaFa baits are scattered are of less concern when considering the inoculum for biodegradation of NaFa. Other physical environmental factors affecting the proliferation or further establishment of induced microflora would be relatively more important. NaFa baits are usually applied in winter when conditions are cold and dry and which may suppress or retard the growth of microorganisms and hence defluorination of NaFa. However, occasional rains and warmer temperatures could

result in a sudden flourish of microbial growth and defluorination of NaFa. In summer, dryness may be the controlling factor in the persistence of residual NaFa. Spring and autumn conditions are generally more favourable to microbial growth, so that NaFa would be less likely to persist for long. Daily monitoring of environmental conditions such as humidity and temperature after NaFa is applied should enable one to estimate the toxicity remaining after some time.

The role of microorganisms in influencing the persistence of NaFa has often been over looked or ignored in preference to studying the effect of temperature and rainfall, thickness and type of baits used. In a detailed study on the effect of weathering of NaFa content of carrot baits of various thicknesses, Staples (1968) found that the disappearance of NaFa was most rapid during decomposition of the baits in the presence of moisture. Both Staples (1968) and Corr & Martire (1971) reported that loss of NaFa from baits was finitely proportional to the amount of rainfall and that a constant amount of NaFa remained in baits, varying from 10-50% depending on the amount of rainfall, type and thickness of baits. Their estimation of NaFa was based on total fluorine analysis, no distinction being made between organic (NaFa-F)F or inorganic F^- so that their estimates of NaFa remaining in the baits could have been the sum of both F^- (from microbial defluorination of NaFa) and NaFa. The loss of residual NaFa from baits after leaching to the soil would mainly be the result of microbial activity, which is dependent on weather to some extent. When NaFa is leached into the soil, pH, moisture and the factors mentioned above would determine the availability of NaFa to microorganisms and hence its persistence.

An ecological concern is raised by the use of NaFa irrespective of its persistence or degradation because one of its degraded products, F^-, when consumed in excess, is toxic The biological link in the ecological food chain resides mainly with plants, some of which may accumulate F^- as camellia does (Peters, 1972), or assimilate F^- and transform it into higher fluoro-organic compounds; this was mentioned briefly in Chapter 1: 1.7 and will be elaborated further in Chapter 5.

3.4.3. Kinetics of Growth and Defluorination

It still remains a matter of conjecture whether growth of filamentous fungi is fundamentally linear or autocatalytic (or exponential). Mandels (1965) maintained that growth of filamentous fungi is non-autocatalytic, unlike single celled organisms with each cell giving rise to 2 daughter cells. However Righelato (1975) considered fungal growth to be mainly exponential. Evidence from literature indicates that both linear and exponential phases of growth exist and persist to varying periods, depending on the growth parameters measured. The rate of individual hyphal extension was constant (linear growth) as reported by Smith (1924) for *Fusarium*, *Pyronema* and *Botrytis* species and by Plomley (1959) for *Chaetomium*. Radial extension of a fungal colony proceeds exponentially until a certain size is reached, 0.1 mm for *Chaetomium* (Plomley 1959), then the increase in growth rate declines until growth becomes linear from colony radius at 3.5 mm to 70 mm. Trinci (1969) also reported that radial colony growth was linear for *Asperigillus nidulans*, *Mucor hiemalis* and *Penicillium chrysogenum*. The work from these authors shows that exponential growth based on measurements of the above parameters was relatively transcient compared to the linear phase of growth. Likewise hyphal density increases exponentially until the maximum number of hyphal apices or growth points is attained. (Plomley, 1959; Trinci, 1969). Mycelial weight when used as a parameter, also shows exponential and linear phases of growth (Trinci, 1969, 1971; Borrow *et. al.*, 1964). Exponential phase of growth has also been shown with protein measurements (Zalokar, 1959).

An ideal growth curve is one which exhibits lag, exponential, decelerating to linear phases of growth followed by a stationary phase. However which phase of growth predominates depends on the parameter used, as mentioned above, the nutrient status of the growth medium and the period of observation. Borrow *et. al.*, (1964), noted that the change from exponential to linear phase of growth of *Gibberella fujikuroi* coincided with nutrient or oxygen limitation. Furthermore, Plomley (1959) stated that exponential growth is maintained by branching which is finite depending on space and nutrients.

In the light of the work done by the above authors, attempts were made to interpret the growth kinetics of *F. solani*

and *A. strictum* in medium with NaFa as the sole carbon source or supplemented with glucose. Where NaFa was the only carbon source supplied growth of *F.solani* and *A. strictum* was fundamentally linear over the period examined, preceded by a lag phase and perhaps a short exponential phase. Linearity of the growth kinetics suggests some factor (or factors) was limiting in the growth medium. Linear growth in NaFa medium may also suggest that NaFa may stimulate and hasten hyphal branching up to a maximum in a very short time so that exponential phase was seen to be transcient or that NaFa may suppress branching. The supression may be relieved in the presence of glucose via its metabolites, one or more of which may regulate or control the process of branching. Growth kinetics of *F. solani* in NaFa medium supplemented with 2.8 mM glucose showed a lag phase followed by exponential, then decelerating to linear phase and a declining to stationary phase. This more pronounced and longer exponential phase was observed only in the presence of glucose and suggests that one of the limiting factors contributing to a linear growth in NaFa medium is carbon, a major component for cell wall synthesis and essential for growth. The decelerating phase could be the result of glucose exhaustion in glucose-supplemented NaFa medium, or limitation of nitrogen Mg^{+} or $PO_4^{\equiv}$. Borrow *et. al*, (1964) suggested that deceleration of growth could be a response to adverse pH. In the present study the pH at the end of incubation period was 6.2, whilst initial pH was 5.8 which is not critical since it was shown previously that the total mycelial yield was the same within this pH range.

The kinetics of defluorination of NaFa by *F. solani* or *A. strictum* exhibited an initial lag phase followed by an accelerating phase within the period examined, and in glucose-supplemented NaFa medium, the acceleration phase continued until all NaFa was degraded. The presence of glucose did not alter the kinetics of defluorination qualitatively but speeded the process. During the period in which growth of the fungi underwent various phases - exponential, decelerating and autolysis, defluorination proceeded at an accelerating rate irrespective of the different phases of growth or state of growth of the fungus even at autolytic state. This implies that defluorination

of NaFa is not dependent on the age of the fungus. Decelerating phase of defluorination would be expected from continually decreasing availability of NaFa, but it was not observed. The amount of defluorinating enzyme synthesised may be small relative to NaFa present and its synthesis may be initiated only at the site to which NaFa was transported if NaFa were actively transported into the mycelium.

3.3.4. Metabolism of NaFa

Defluorination of NaFa has been shown to be a biological process catalysed by an enzyme which was found to be intracellular in *F. solani*, from present work. That the enzyme was intracellular was previously shown in the bacteria, *Pseudomonas* sp. (Tonomura *et. al.*, 1965) and presently from studies of the activity of the culture fluid, and from fractionated cell-free extracts. Fluoroacetate is a small molecule. It is not known whether it was actively transported into the cell or enters by diffusion.

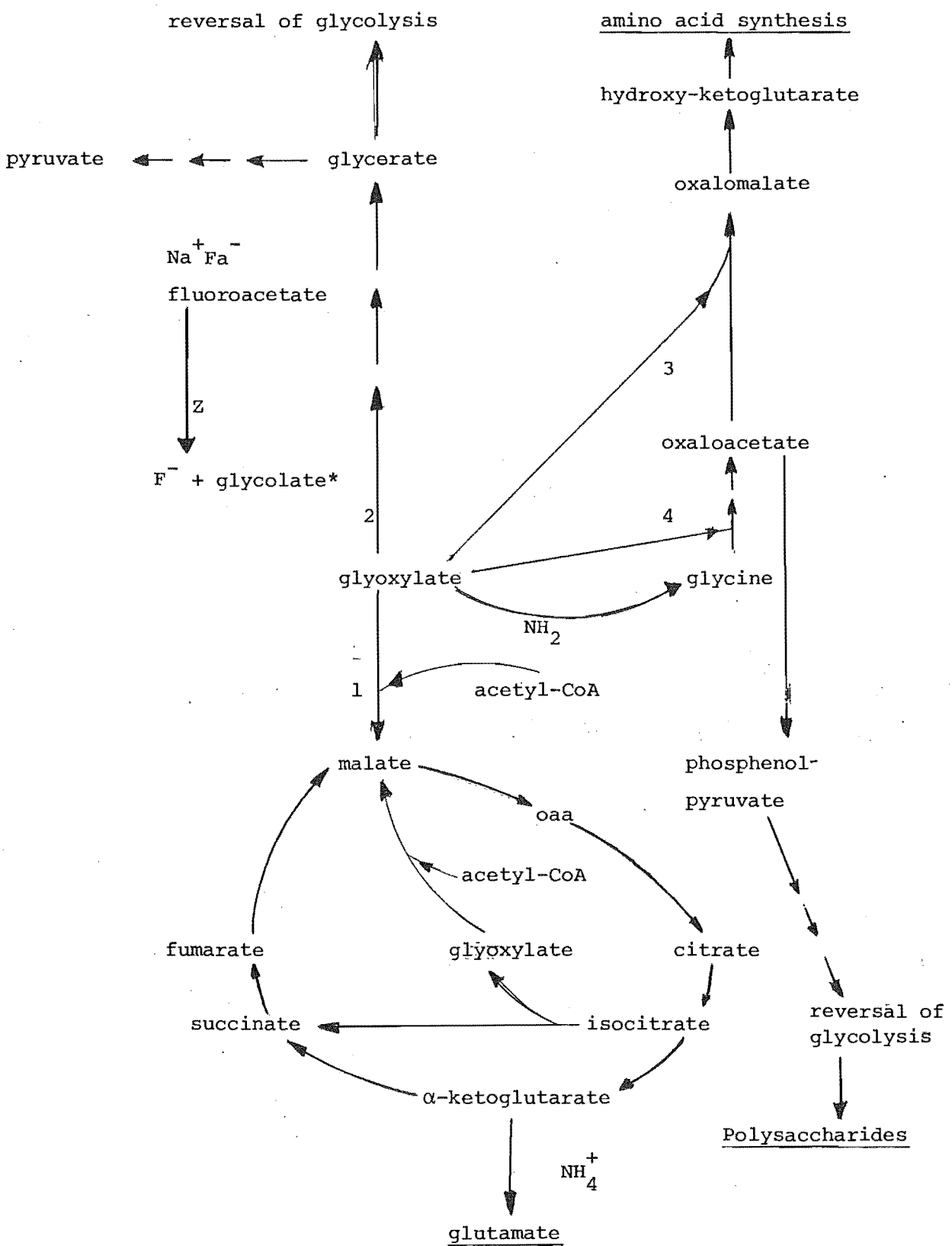
Once inside the cell, fluoroacetate is defluorinated to yield inorganic F^- and glycolate as identified by Goldman (1965), and Tonomura *et. al.*, (1965) and confirmed in the present study. Inorganic F^- was rapidly excreted into the external medium and in a medium where NaFa was the only carbon substrate available, growth occurred indicating that glycolate was being metabolised and assimilated. There are several possible routes of entry of glycolate into the respiratory and biosynthetic pathways of the organism, all of which involve its initial oxidation to glyoxylate catalysed by glycolate oxidase. Glyoxylate is an extremely reactive compound and its biochemical reactions have been reviewed by Wegener *et.al.*, (1968). It may condense with another glyoxylate molecule yielding tartronic semialdehyde plus CO_2 and the condensation is catalysed by a thiamine phosphate - dependent glyoxylate carboligase. Tartronic semialdhyde is subsequently reduced to glycerate by a reductase. Both enzymes are induced in bacteria grown on glycolate. Another mechanism is the direct amination or transamination of glyoxylate to glycine which then condenses with another molecule of glyoxylate to eventually yield oxaloacetate. This pathway has been demonstrated in *Micrococcus denitrificans*. Oxaloacetate could further condense with glyoxylate to form oxalomalate which can be decarboxylated to form hydroxy-ketogularate important in glutamate synthesis.

This pathway was found in *Acetobacter suboxydens*. Alternatively, the oxaloacetate so formed could be decarboxylated to phosphoenolpyruvate to be used in gluconeogensis as found in fungi grown on acetate (Berry, 1975). A summary of glycolate metabolism is presented in Figure 3.10.

It was found that in the presence of lower concentrations of acetate ($\leq$ 10 mM) or glucose ($\leq$ 5 mM), the amount of NaFa degraded was increased and growth of *Fusarium solani* was enhanced. The stimulation could have been the result of availability of acetyl CoA from acetate or glucose, to condense with glyoxylate derived from NaFa, a reaction catalysed by malate synthase. An essay for malate synthase activity has not been attempted in present work, so this remains a suggestion. It is known however, that malate synthase is induced by acetate or glyoxylate in *Rhizopus nigricans* grown on, or transferred to acetate or glycolate medium (Wegener *et al.*, 1968).

In the presence of higher concentration of acetate, NaFa defluorination was reduced whilst growth continued to be enhanced indicating that acetate suppressed defluorinating activity, probably by competitive inhibition, which is concentration-dependent. There are several possible sites of competition: one of which is competition for the carrier molecule(s) involved in transporting the substrates, and the other in the metabolic pathway. The former has been mentioned earlier. Both acetate and glycolate metabolism involves the intermediate glyoxylate. Studies on *Rhizopus nigricans* (Wegener *et. al.*, 1968) showed that acetate stimulates glyoxylate cycle operation and its characteristic enzymes are isocitrate lyase and malate synthase. Growth of the fungus on glycolate stimulates synthesis of a malate synthase which is distinct from the malate synthase induced by acetate in that the former is more thermally stable and its synthesis is not regulated by Zn^{++}. "Glycolate-induced" malate synthase serves mainly a respiratory function whereas "acetate-induced" malate synthase functions chiefly in replenishing C_4 acids to TCA cycle (Wegener *et. al.*, 1968). In the presence of both acetate and NaFa in comparable concentrations, it is not known how these forms of synthase would interact. If competition exists, glyoxylate cycle operation would be favoured by high acetate concentrations or suppressed in low acetate concentrations, and the metabolism of glyoxylate from NaFa would be affected.

FIG 3.10 Proposed pathways for metabolism of NaFa by *F. solani*



oaa: oxaloacetate Possible routes of entry glycolate* via glyoxylate into the respiratory, 1 & biosynthetic pathways, 2,3, 4, 5.

fluoroacetate enters the organism where it is defluorinated, Z; F^- is released and leaves the organism.

Thus NaFa defluorinating activity may be so regulated by end-product inhibition or by demands for C_4 intermediates.

NaFa defluorination by *F. solani* is also affected by glucose in a concentration-dependent manner, stimulation at low concentration of glucose and "de-stimulation" at higher concentration. The presence of high glucose concentration would create a situation whereby supply of C_4 acids could meet the demand so that utilization of glycolate from NaFa breakdown is not required. At low glucose concentrations, the situation is reversed so that a stimulation in NaFa breakdown was observed.

NaFa breakdown by *Penicillium* sp increased proportionally with increasing glucose concentration, unlike the response of *F. solani*. A difference between *Penicillium* sp and *F. solani* is that the former is unable to defluorinate NaFa in the absence of glucose. That NaFa breakdown by *Penicillium* sp. occurs only in the presence of glucose suggests that glucose is required to provide the initial carbon substrate for growth and synthesis of the defluorinating enzyme. A further difference is the response to increased aeration of the medium. NaFa breakdown by *Penicillium* sp. was not affected by increased aeration whereas that by *F. solani* was reduced. It would seem that glucose metabolism by glycolysis or the TCA cycle is not affected by the presence of glycolate or else that NaFa breakdown is independent of the state of respiration of *Penicillium* sp. In *F. solani*, the TCA cycle could be favoured by higher aeration, and increased metabolism of glucose. In a low aeration status, the TCA cycle would be less active and a partial supply of C_4 acids may come via glyoxylate from NaFa breakdown.

Subsequent metabolism of NaFa after it is defluorinated requires further investigation as does the question of whether fluoroacetate can be assimilated *per se* by micro-organisms. Studies with *F. solani* showed that NaFa could be completely defluorinated, indicating that fluoracetate was not assimilated as such by this fungus. On complete defluorination, all the inorganic F^- was found in the growth medium showing that it was not metabolised by the fungus. Whether inorganic F^- would be metabolised after prolonged incubation is not known. Certain fungi are known to metabolise halides. Foster (1949) and Cochrane

(1958) cite a number of secondary metabolites containing chlorine as a constituent: for examples, griseofulvin and nalgiolaxin produced by species of *Penicillium*, caldariomycin and terrein produced by *Caldariomyces fumago* and *Aspergillus terreus* respectively, and sclerotiorine by *P. sclerotiorum*. As yet the synthesis of fluoro-organic compounds from F^- by micro-organisms have not been reported. The ability of micro-organisms to transform fluoroacetate to fluorocitrate (Black & Hutchens, 1948) or fluorohexanoic compounds (Aldous, 1963) has been demonstrated in yeasts, in *Vibrio* 01 (Dagley & Walker, 1956), and possibly to fluoromalate (Callely & Dagley, 1959), and in *Pseudomonas* OD1 (Jayasuriya, 1956). Twenty other examples of the microbial transformation of cyclic fluoro-organic compounds have been listed by Kieslich (1976).

CHAPTER FOUR

BIOCHEMICAL ASPECTS OF NAFA DEFLUORINATION

4.1 INTRODUCTION

The defluorination of NaFa by growing organisms has been established earlier in Chapter 3. In the following studies, attempts were made to compare the biochemical aspects of NaFa defluorination by resting cells, and defluorinating enzyme preparations from *F. solani* and *Pseudomonas* sp. The enzyme was named haloacetate halidohydrolase by Goldman & Milne (1966), and it catalysed the defluorination of NaFa to F^- and glycolate as shown:

$$\text{fluoroacetate} + H_2O \xrightarrow[\textit{halidohydrolase}]{\textit{haloacetate}} F^- + \text{glycolate}$$

A more colloquial name for this enzyme is fluorohydrolase. Attempts were also made to further characterise fluorohydrolase in terms of molecular weight, stability, substrate specificity, response to pH and temperature.

4.2 MATERIALS AND METHODS

4.2.1 Culture and Harvest of Organisms

Pseudomonas sp. was cultured in 40 mM NaFa mineral salts medium (ph 6.8), as previously described (2.2.3), at 25°C for 2 days. Bacterial cells were harvested by centrifugation at 5,000 g for 10 mins. *Fusarium solani* was grown in 40 mM NaFa medium supplemented with 2.8 mM glucose, pH of the medium was 5.8. Fungal cultures were incubated at 25°C for 7-10 days and mycelium collected by filtering through a double layer of muslin cloth.

The bacterial cells or mycelium were washed twice with phosphate buffer (pH 7.0) within 24 h and stored at -20°C or freeze-dried.

4.2.2. Preparation of Cell-free Extracts

Bacterial cells or mycelium were suspended in two volumes of phosphate buffer (pH 7.0) and disintegrated using a Bronson model Untrasonicator at 20 KHz for 10 - 15 minutes. The operation was carried out in the cold room (6°C) and the temperature of the cell suspension was kept below 10°C by sitting the container in an ice bath. These suspensions were then centrifuged at 25,000 g for an hour at 4°C. Cell-free supernatant was decanted, the cell debris is re-suspended in the same buffer, ultrasonicated again; centrifuged and the supernatant pooled with

the original extract. Protein concentration and defluorinating activity of the extracts were assayed immediately prior to being stored at -20°C or freeze-dried.

4.2.3. Analytical Methods:

a. All assays were performed in triplicate. The standard reaction mixture consisted of 1.5 ml 20 mM NaFa (unless otherwise specified) and 1.5 ml enzyme extract or intact cell suspension. Appropriate controls were prepared with 1.5 ml phosphate buffer in place of NaFa, or 1.5 ml boiled enzyme extract or cell suspension. Reaction mixture was incubated at 25°C for a specified period.

b. Protein concentration was estimated by the colorimetric method of Lowry *et al.*(1951) using crystalline egg albumen made up in phosphate buffer (pH 7.0) as protein standard. Protein formed a colored copper complex, the color was read on EEL colorimeter, using a 608 red filter.

c. Inorganic F^- was determined using the F^--specific electrode. The electrode was calibrated with NaF standard made up in phosphate buffer (pH 7.0).

d. Glycolate was determined using the method of Lewis and Weinhouse (1957) previously described in Section 3.2.4.

4.2.4 Procedures Used in Enzyme Purification

a. Heat Treatment - Proteins vary in their thermal stability and this may be used to fractionate mixtures. The crude enzyme extract of *Pseudomonas* sp. was incubated at 55°C for an hour and resultant precipitate was removed by centrifuging at 25,000 g for 10 mins. This procedure was not suitable for use with extracts of *F. solani*.

b. Fractionation with ammonium sulphate - Solid $(NH_4)_2SO_4$ was added whilst stirring the supernatant collected from above. Saturation of the supernatant was increased at 5-10% intervals. The fractions precipitated were removed by centrifugation (25,00 g for 15 min) after standing the preparation for 1 h. Precipitates were dissolved in a minimal volume of phosphate (pH 7.0) or Tris-HCl buffer (pH 7.6). These were then dialysed against the respective buffer to remove $(NH_4)_2SO_4$. Fractions

with highest specific activity were pooled for subsequent analysis.

c. Sephadex Gel-filtration - Gel-filtration separates proteins on a basis of molecular weight. Sephadex gels of specified grades were washed with phosphate buffer (pH 7.0) and soaked in the same buffer for 1-3 days depending on the grade. The gel suspension was de-aerated prior to pouring it into a column (25 x 340 mm) and enzyme solution (2 ml) in 200 mM sucrose, prepared as above was applied to the top of the column. Sephadex gels G-75, G-100, and G-150 were evaluated.

d DEAE-cellulose column chromatography - Pre-swollen Whatman DEAE-cellulose No 52, washed and equilibrated with Tris-HCl buffer (ph 7.6) was poured into a column (15 x 250 mm). The sample in 5 ml of 0.2 mM sucrose was applied to the top of the column, and eluted with Tris-HCl buffer after a short period of adsorption of proteins to the ion-exchanger. Stepwise elution with increasing concentrations of NaCl in Tris-HCl buffer followed. The fractions eluted by each concentration of NaCl were either dialysed against Tris-HCl or phosphate buffer or desalted by gel-filtration on a Sephadex G-25 column previously equilibrated with phosphate buffer.

4.2.5. Molecular Weight Determination

Molecular weights of defluorinating enzymes were estimated by molecular-sieve chromatography on a Sephadex G-150 column (25 x 340 mm). The stability of the column was checked by repeatedly eluting Blue Dextran through until a constant void volume (V_o) was obtained. The column was subsequently calibrated with proteins of known molecular weights - 45,000 (monomer of egg albumen); 68,000 (haemoglobin) and 134,000 (serum albumen), 1.0 ml of protein standard (5 mg ml^{-1} of each protein) was eluted through the column. 1.0 ml aliquots of bacterial and fungal cell-free extracts (from the ammonium sulphate fractionation) were eluted separately through the same column and fractions collected assayed for protein and enzyme activity.

Where column chromatography was used, an ISCO model

UA-4 UV-monitor set at 280 nm was used to monitor the protein elution pattern.

4.2.4e. Polyacrylamide disc gel-electrophoresis - The purity of the various fractions was examined by electrophoresis on 5% polyacrylamide (Cyanogum 41) gels prepared as described by Sargent & George (1975). Protein samples were applied in 1 M surcrose to the top of the gel using a syringe: 6 gels were run for each protein sample under constant current (4 mA per gel) for 1½ h at 6°C. Proteins were located by staining with Amido Black 10B as described by Sargent & George (1975).

Attempts were made to localise the enzyme (fluorohydrolase) on the gels indirectly be detection of a zone of F^- release band. Gels were incubated in 20 mM NaFa (phosphate buffer, pH 6.8) for 30 mins at 25°C. Attempts to detect F^- released into the gels at the site of the enzyme were by:

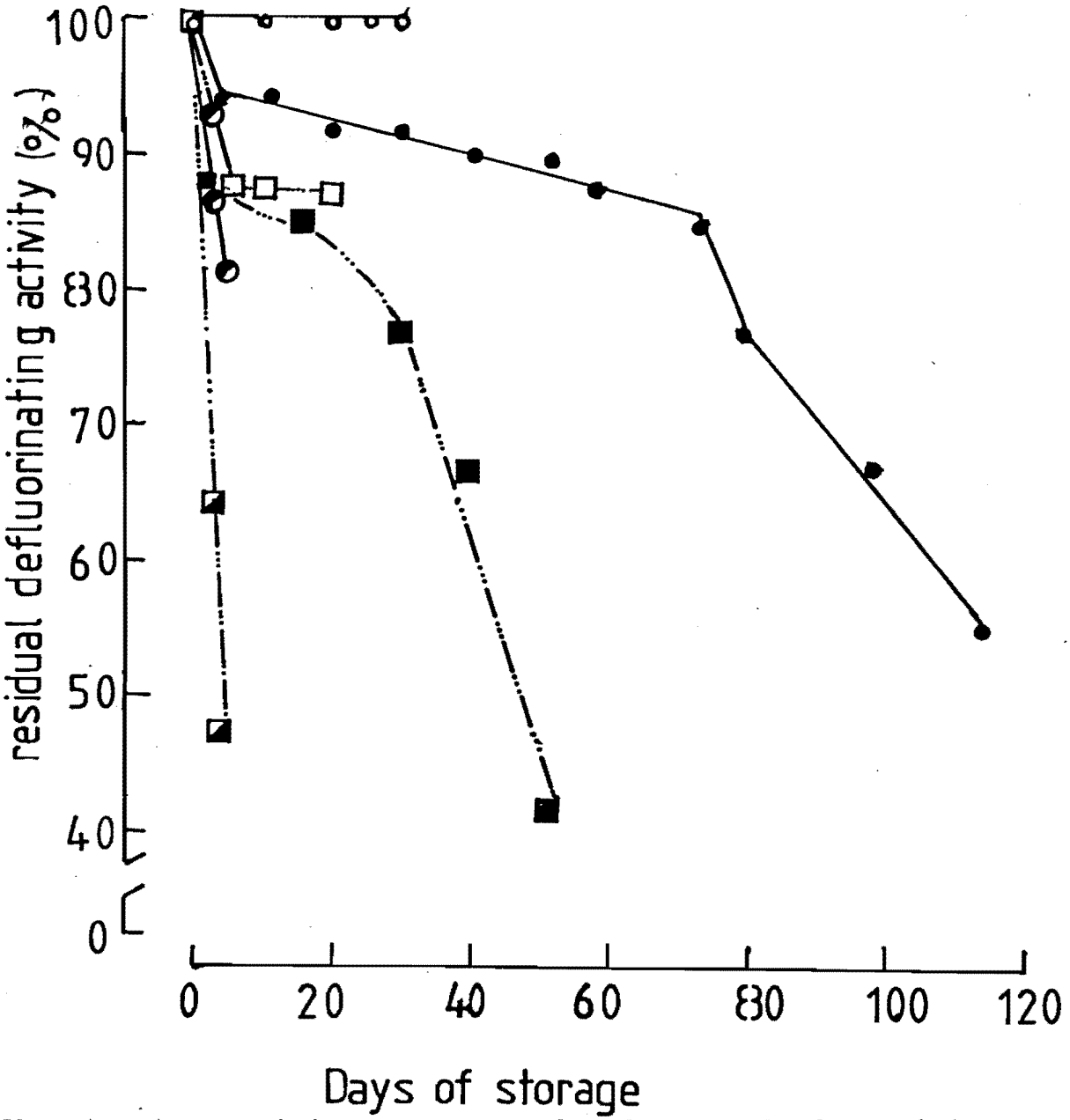
(a) Soaking the incubated gels in $ZrO^=$ - SPADNS solution, the F^- band should appear less pink; or

(b) Soaking the incubated gels in $Ca(NO_3)_2$ solution to detect a zone of CaF_2 precipitation in the gel.

4.3.1 Stability of Defluorinating Enzymes of *Pseudomonas* sp. *F. solani*

The defluorinating activity of crude enzyme extracts of *Pseudomonas* sp. and *F. solani* at 6°C, - 20°C, and freeze-dried form stored at room temperature (18-22°C), was assayed over a period of months. Enzyme extracts were made up in pH 7.2 phosphate buffer, and assayed at the same pH. Stability of the extracts was assessed from their residual ability to defluorinate NaFa. The bacterial enzyme was more stable compared to the fungal enzyme at 6°C, - 20°C and in freeze-dried form, as shown in Figure 4.1. Freezing resulted in a reduction of 13% in specific activity of fungal enzyme whilst the bacterial enzyme was unaffected. In freeze-dried form, loss of activity was most rapid after 20 days of storage for fungal and bacterial enzyme respectively. Heat tolerance of crude enzyme entracts of *Pseudomonas* sp. and *F. solani* was tested by preincubating each extract at different temperatures for various times as specified in Table 4.1. Protein precipitates were removed by centrifugation at 10,000 g for 10 min, redissolved in phosphate

FIG 4.1: STABILITY OF FLUOROHYDROLASE (CRUDE EXTRACT) OF *Pseudomonas* SP AND *F. solani* DURING STORAGE



Defluorianting activity was assayed under standard conditions: reaction mixture contained 1.5 ml 20 mM NaFa solution and 1.5 ml crude enzyme solution (pH 7.2), incubated at 25°C for 30 min, and F^- released measured. Results are means of triplicates.

CONDITIONS OF STORAGE		Enzyme extracted from: *Pseudomonas* sp.	Enzyme extracted from: *F. solani*
pH 6.8 (20 mM PHOSPHATE BUFFER)	6°C	●—●	◪···◪
pH 6.0	- 20°C	○—○	□···□
(SAME AS ABOVE)			
FREEZE DRIED		—●	···■

buffer, and protein concentrations standardised before being assayed for defluorinating activity. Results are shown in Table 4.1. Activity was detected in both supernatant fractions but not the precipitates. The defluorinating enzyme (henceforth termed as fluorohydrolase) from *Pseudomonas* sp. in supernatant fraction showed an increase in specific activity whereas that from *F. solani* was totally inactivated after 1 h incubation at 55°C. Both fluorohydrolases were inactivated above 78°C. The results thus show that the fluorohydrolase of *Pseudomonas* sp. was thermally more stable than that of *F. solani*.

Table 4:1 Thermal Sensitivity of Defluorinating Enzyme Extracts of *Pseudomonas* sp. and *F. solani*.

Temperature	Preincubation Period	Relative Defluorinating Activity			
		Pseudomonas sp.		*F. solani*	
		Supernatant	Precipitate	Supernatant	Precipitate
0°C	24 hours	1.0	-	0.9	-
25°C	60 mins	1.0	-	1.0	-
30°C	60 mins	1.1	-	1.0	-
40°C	60 mins	1.5	-	0.8	-
55°C	60 mins	2.0	0.06	0.0	0.0
78°C	20 mins	1.94	0.0	0.0	0.0

Reaction mixture (1.5 ml enzyme solution + 1.5 ml 20 mM NaFa) incubated at 25°C for 30 min. F^- released then measured.

4.3.2a. Purification of fluorohydrolases from *Pseudomonas* sp. and *F. solani*

Heat treatment - Consequent upon the above finding that the fluorohydrolase of *Pseudomonas* sp was stable at 55°C, the crude enzyme extract was accordingly heated. This resulted in 1.5-fold increase in specific activity with a recovery of 89%.

$(NH_4)_2SO_4$ fractionation - The supernatant obtained from above was fractionated with $(NH_4)_2SO_4$ at 5-10% saturation intervals. Highest specific activity was obtained from the fraction between 40-50% saturation with a 5.8-fold increase in activity and 50% recovery. The crude enzyme extract from *F. solani* was similarly fractionated with $(NH_4)_2SO_4$. Fractions precipitated between 30 and 65% saturation were pooled, yielding a 2.5-fold increase in activity with 96% recovery.

Gel-filtration - Fractions collected from above were eluted through a column of Sephadex G-75 with 20 mM phosphate buffer. The elution pattern is shown in Figure 4.2a. Active fractions from *Pseudomonas* sp. and *F. solani* were eluted in a similar volume, indicating a similarity in molecular weight of fluorohydrolase from different sources. The recovery of activity from this step was only 8.8% and 7% for *Pseudomonas* sp. and *F. solani* extracts, with 6.6- and 4.2-fold increase in activity respectively.

DEAE-Cellulose 52 Chromatography - fractions of *Pseudomonas* sp. extract after $(NH_4)_2SO_4$ fractionation were eluted through a DEAE cellulose column. Active fractions were eluted with 100 mM NaCl, with 8-fold increase in activity and recovery of 9%. Elution pattern is shown in Figure 4.2b.

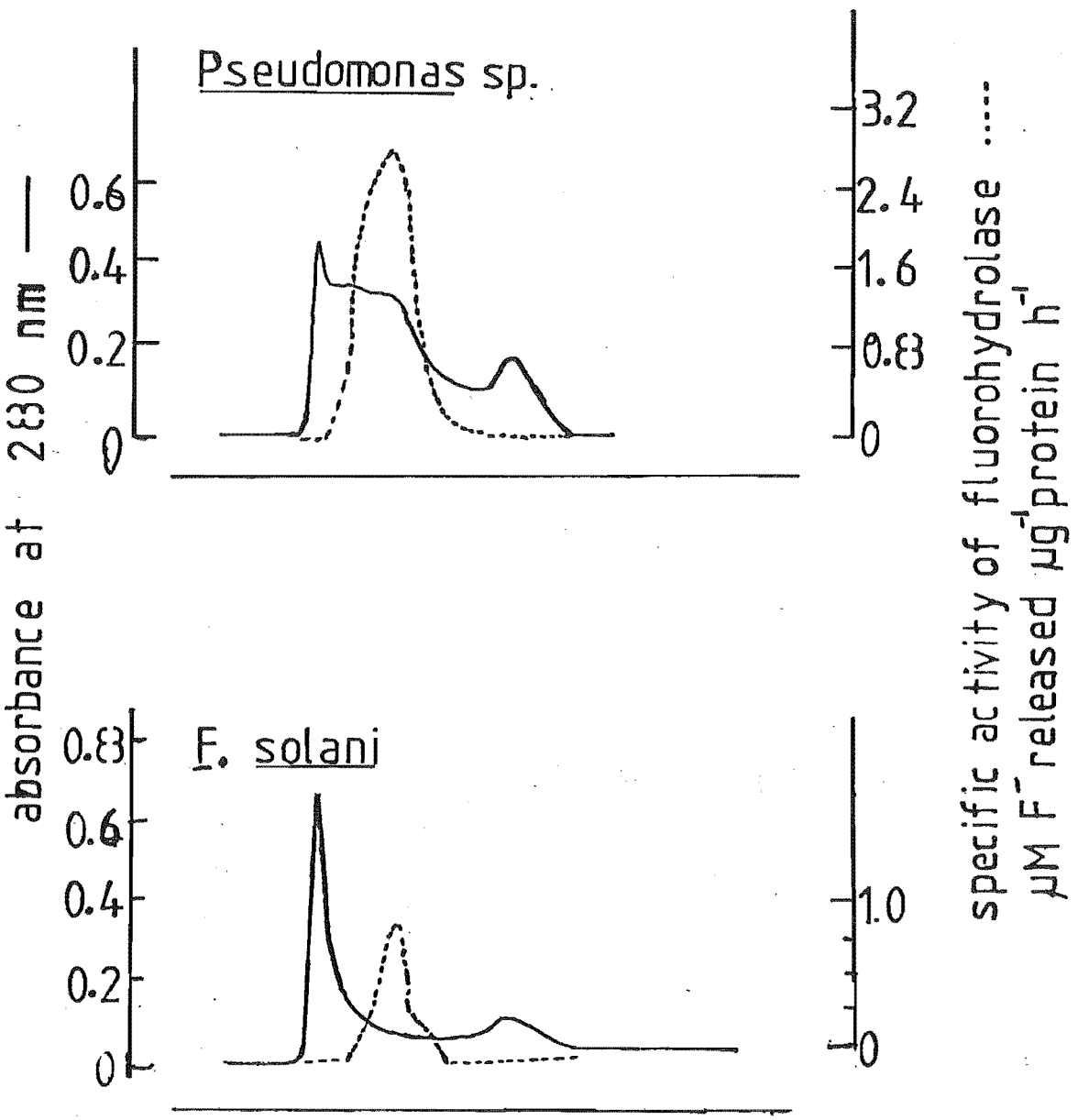
Purity of various fractions collected from above steps: - The purity of the various fractions was assessed from the number of protein bands detected on gels after electrophoresis. The number of protein bands decreased with each successive purification step as shown in Table 4.2. Insufficient protein was recovered from elution through either Sephadex G-75 or DEAE cellulose 52 column to allow examination of the purity of these eluates.

A summary of these results of the various purification procedures is given in Table 4.2. Comparison of the protein and activity curves shows that they do not coincide suggesting that the actual enzyme concentration in the extracts was not high enough to permit detection by either the UV monitor or disc-gel electrophoresis. Attempts to localise fluorohydrolase activity on gels with $ZrO^{=}$ -SPADNS reagents were not successful - no clear F^{-} band appeared on gels previously incubated with NaFa. No decolorisation occurred on $ZrO^{=}$-SPADNS treated gels while gels soaked in $Ca(NO_3)_2$ solution were uniformly opaque.

4.3.2b Molecular Weight of fluorohydrolase from *Pseudomonas* sp. and *F. solani*.

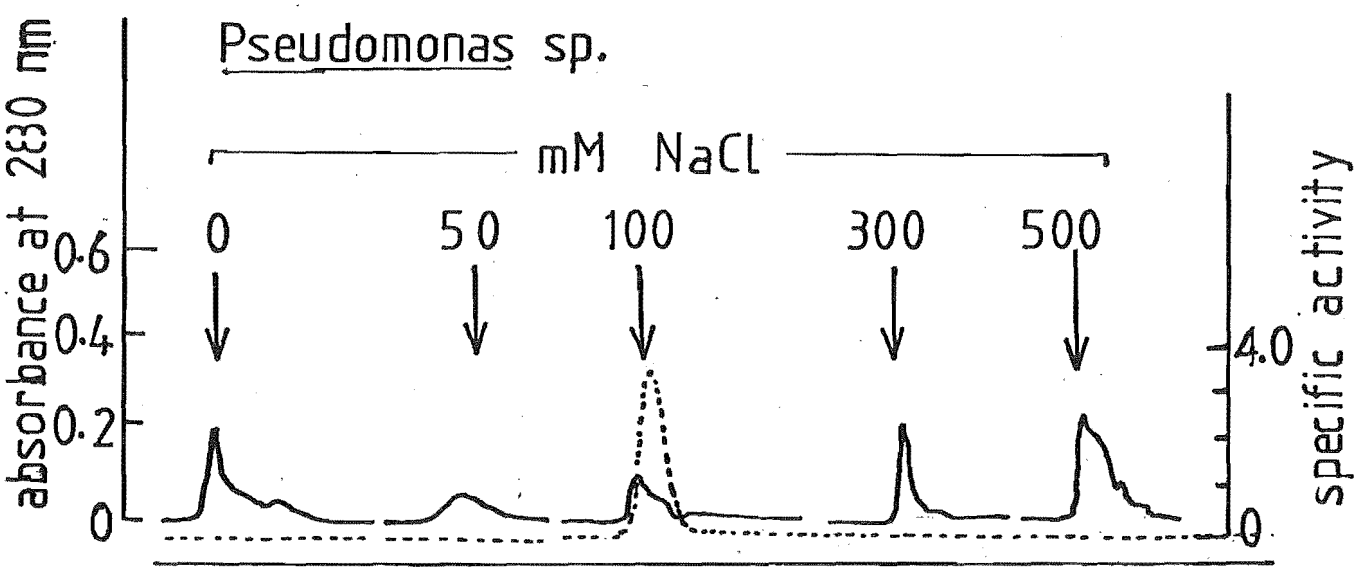
Because an insufficient quantity of reasonably pure fluorohydrolase fraction was recovered, the partially purified fraction after $(NH_4)_2SO_4$ precipitation was used for determining molecular weight by chromatography on a column of Sephadex G-150 previously calibrated with protein standards.

FIG 4.2A GEL-CHROMATOGRAPHY OF FLUOROHYDROLASE ON SEPHADEX G-75 COLUMNS



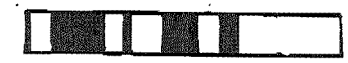
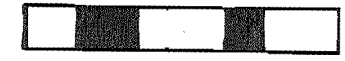
Sephadex G-75 column (25 x 340 mm) was equilibrated with 20 mM phosphate buffer (pH 7.0). Protein sample was eluted with the same buffer at the rate of 150 ml h^{-1}. Specific activity of each fraction collected was assayed under conditions described in fig 4.1.

FIG 4.2B: CHROMOTOGRAPHY OF FLUOROHYDROLASE ON DEAE - CELLULOSE 52 COLUMN.



DEAE 52 cellulose column (15 x 250 mm) was equilibrated with 20 mM Tris-HCl buffer (pH 7.6). Protein was eluted with NaCl in increasing concentration in a stepwise manner at the rate of 108 ml h^{-1}. Fractions collected were dialysed against pH 7.0 phosphate buffer prior to assay of specific defluorinating activity under conditions described in Fig 4.1.

Table 4.2: Purification of Defluorinating Enzyme of *Pseudomonas* sp.

Steps	Total Protein (mg)*	Specific Activity	Total Activity	Yield %	Purification	Electrophoresis
Crude	1.2	0.41	492	100	1	6 protein bands 
Heating at 55°C	0.7	0.63	440	89	1.5	
$(NH_4)_2SO_4$, 30% - 70% (Pooled fractions)	0.2	1.23	245	50	3.0	4 protein bands 
Sephadex G-75	0.016	20.72	43.5	8.8	6.6	Insufficient protein for electrophoresis
or						
DEAE-Cellulose 52	0.013	3.32	43.2	8.8	8.1	Insufficient protein for electrophoresis
Purirification of Defluorinating Enzyme of *Fusarium solani*						
Crude	0.60	0.22	132	100	1	4 protein bands 
$(NH_4)_2SO_4$, 30% - 65%	0.24	0.53	127	96	2.5	2 protein bands 
Sephadex G-75	0.01	0.92	9.2	7.0	4.2	

Specific activity is defined as μM F^- released μg^{-1} protein h^{-1}. * Protein concentration is determined by the method of Lowry *et. al.* (1951).

The molecular weight of the enzyme was deduced from the enzyme activity curve rather than from the elution profile. Active fractions of both bacterial and fungal enzyme preparation were eluted at the same position in the 45,000-68,000 molecular weight region as shown in Figure 4.3.

4.3.3. Stoichiometry of NaFa Defluorination

The stoichiometry of the enzymatic conversion of NaFa to F^- and glycolate was demonstrated earlier by Goldman (1965) using an enzyme preparation from a soil pseudomonad. His experiment was repeated here with partially purified enzyme preparations [$(NH_4)_2SO_4$ fractionation] from *Pseudomonas* sp. and *F. solani*. The results are presented in Table 4.3, showing that glycolate and F^- were present in similar molar concentrations in reaction mixtures containing both enzymes from *Pseudomonas* sp. and *F. solani*.

Table 4.3 Stoichiometry of Enzymatic Defluorination of NaFa

Reaction Mixture	Product yield (mM); extract from :			
	F. solani		*Pseudomonas* sp.	
	F^-	glycolate	F^-	glycolate
10 mM NaFa +	0.08	0.0	0.2	0.0
boiled enzyme	0.08	0.0	0.2	0.0
10 mM NaFa +	0.76	0.74	5.0	4.8
enzyme	0.80	0.76	5.0	5.0
	0.80	0.80	5.0	4.8

Reaction mixture incubated at 25°C for 30 mins.

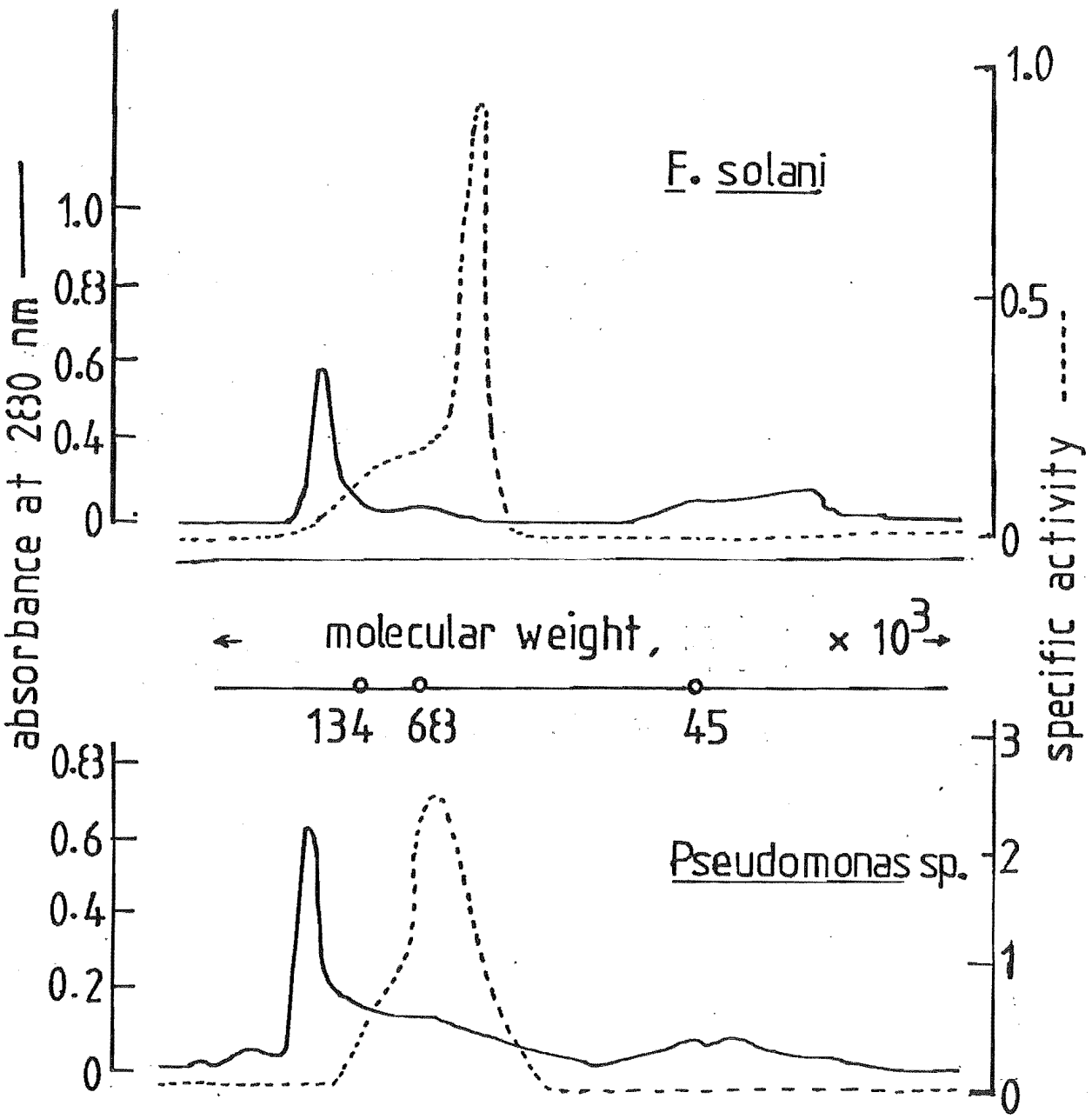
4.3.4. Effect of pH and Temperature

Three buffer systems (McKenzie, 1969) were used to cover the pH range, 4.3-10.4; these were:

Citric acid/phosphate buffer - pH 4.3-7.6;
Sodium phosphate buffer - pH 5.0-8.0; and
Glycine/NaOH buffer - pH 8.6-10.4.

The effect of pH was studied at the cellular level using resting bacterial cells and at enxymatic level using crude enzymes from *Pseudomonas* sp. and *F. solani*. The initial velocity of defluorination was the parameter measured. The

FIG 4.3: DETERMINATION OF THE MOLECULAR WEIGHT OF FLUOROHYDROLASE, USING A PRE-CALIBRATED SPEHADEX G-150 COLUMN



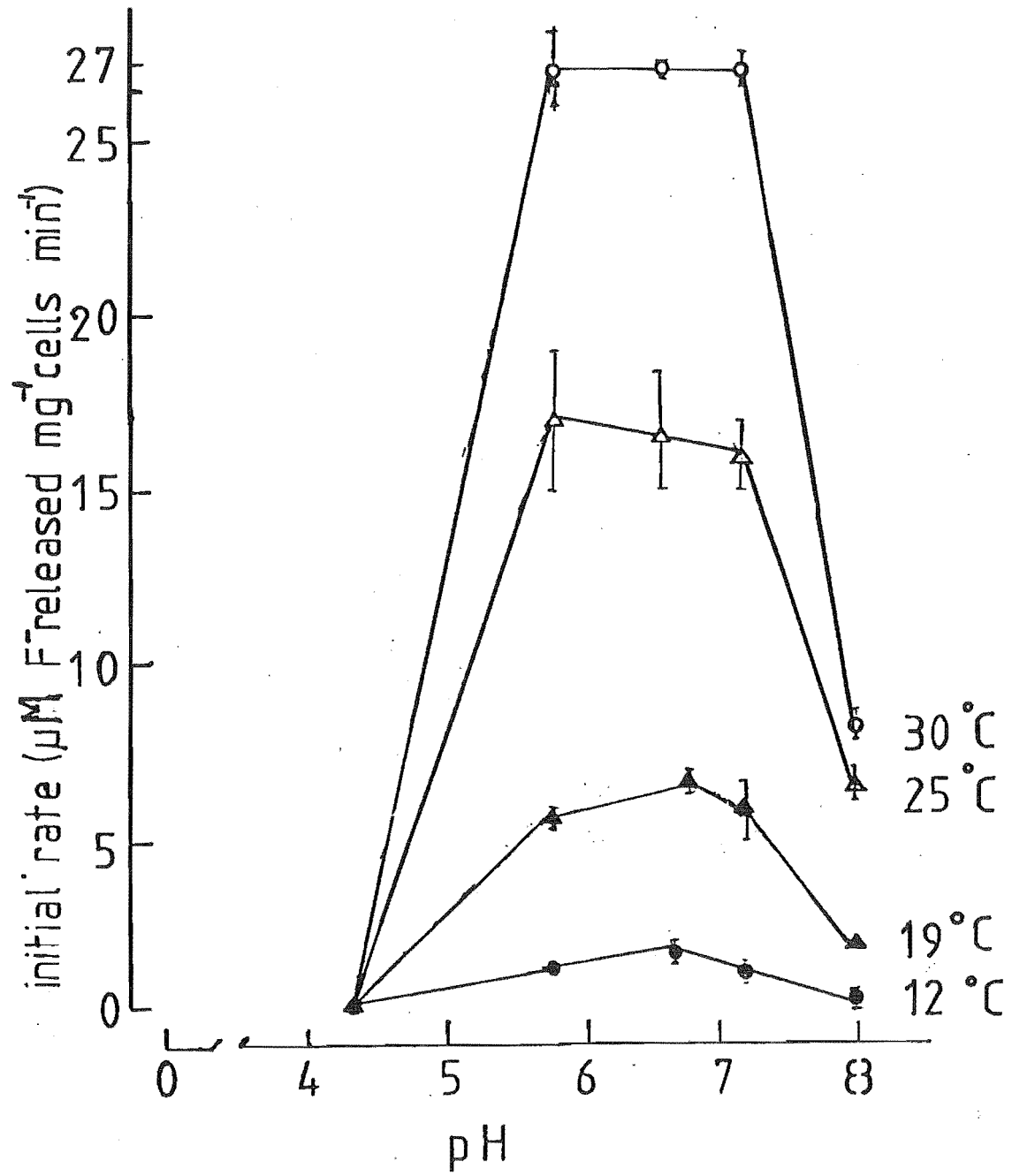
Sephadex G-150 column (25 x 340 mm) was pre-calibrated for molecular weight determination with protein standards: egg albumen (45,000) haemoglobin (68,000), and serum albumen (134,000). Fluorohydrolase fractions prepared from ammonium sulphate fractionation were eluted with phosphate buffer.

effect of pH on defluorination of NaFa by resting cells at various temperatures is shown in Figure 4.4a. Within the temperature range, 12-30°C. Defluorination of NaFa was not detected at pH 4.3 optimal pH plateau (highest defluorinating activity) at pH 5.8-7.2 was observed. Enzymatic defluorination of NaFa showed a narrower optimal pH range, 7.6-8.0 for fluorohydrolase from *F. solani* and at pH 8.0 for bacterial fluorohydrolase as depicted in Figure 4.4b. The enzymes were more sensitive to pH below the optimal range than to pH above 8.0. No activity was detected at pH $\leq$ 5.0. The effect of low pH on the activity of fluorohydrolase of *Pseudomonas* sp. was studied further. The enzyme was exposed to a pH range of 3.6-5.8 for 15 mins and then readjusted to pH 7.2 with NaOH and activity subsequently assayed: activity was restored to the acid-treated enzyme when the pH was returned to 7.2.

The effect of temperature on the rate of defluorination of NaFa by bacterial cells is shown in Figure 4.5a at various pH's. The rate increased with increasing temperature, more sharply at lower temperature range (12-25°C) than at the higher temperature range (25-30°). Temperature coefficient (Q_{10}), the factor by which the rate of defluorination increases for every 10°C increase in temperature, was highest at pH 8.0(10.6), and lowest at pH 6.6 (5.3), and at pH 5.8 and 7.6, Q_{10} was 7.3 within the temperature range, 12-25°C. The temperature coefficient declined for temperatures above 25°C at all pH and more markedly at pH 8.0 as indicated by the gentler slope (Figure 4.5a). The effect of temperature on the rate of defluorination of NaFa by fluorohydrolase of *Pseudomonas* was assayed at pH 7.2, and was shown using an Arrhenius plot (Figure 4.5b). The effect on the enzymatic and on cellular defluorination of NaFa was qualitatively similar. However the Q_{10} of enzymatic defluorination was much lower, 1.93 for temperature range of 15°C - 32°C, and 1.3 between 32°C and 50°C.

To calculate the energy of activation (E_A) of the fluorohydrolase-fluoroacetate complex, Arrhenius equation (IV) was used (Dixon & Webb, 1964):

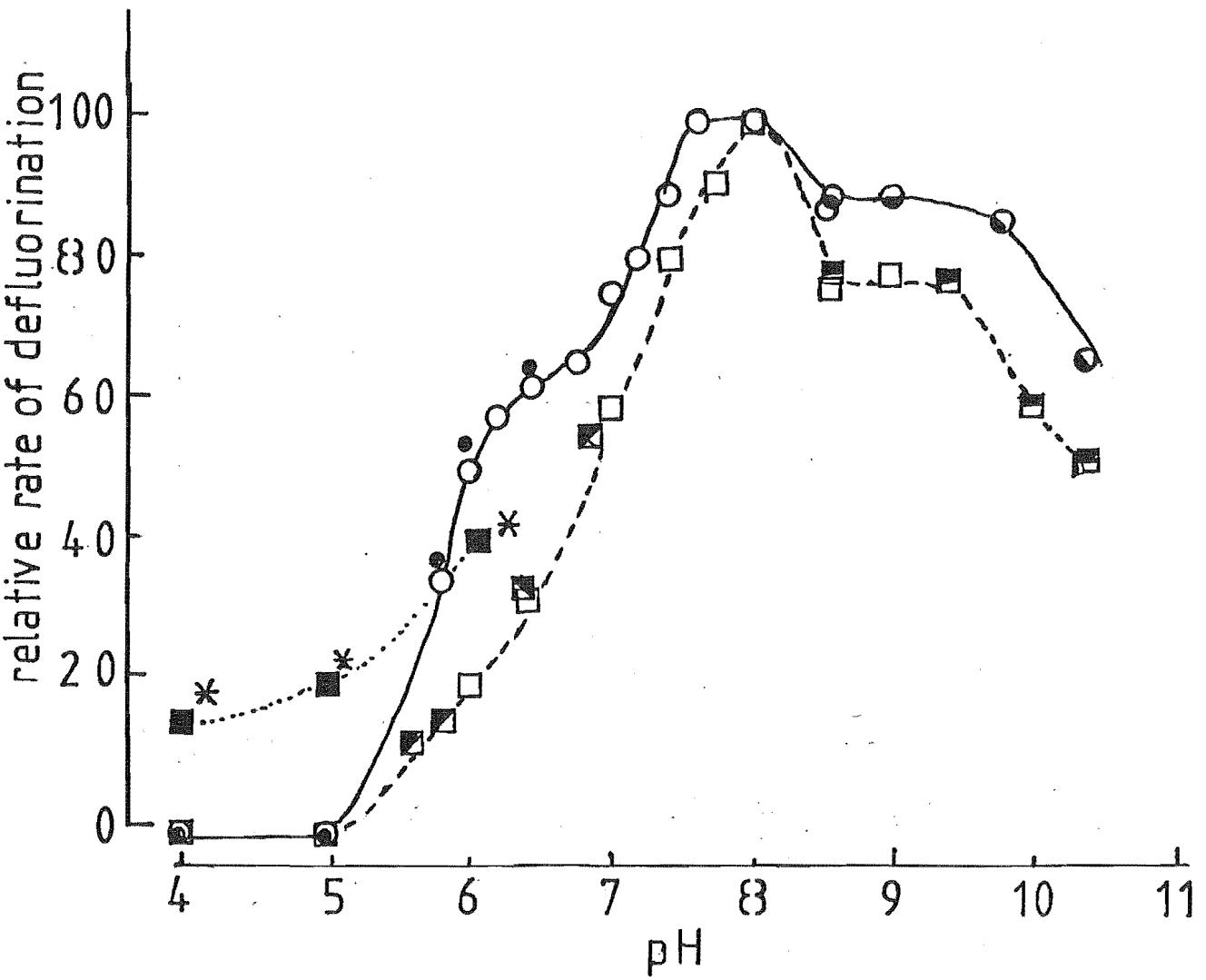
FIG 4.4A EFFECT OF pH AND TEMPERATURE ON THE RATE OF DEFLUORINATION: RESTING CELLS OF *Pseudomonas* SP.



Defluorinating activity was assayed at the pH and temperature indicated under standard conditions as described in Fig. 4.1 F^- released was measured at intervals for 30 min. Results are means of triplicates.

FIG 4.4 B

EFFECT OF pH ON THE ACTIVITY OF FLUORODYDROLASE FROM *Pseudomonas* SP. AND *F. solani*



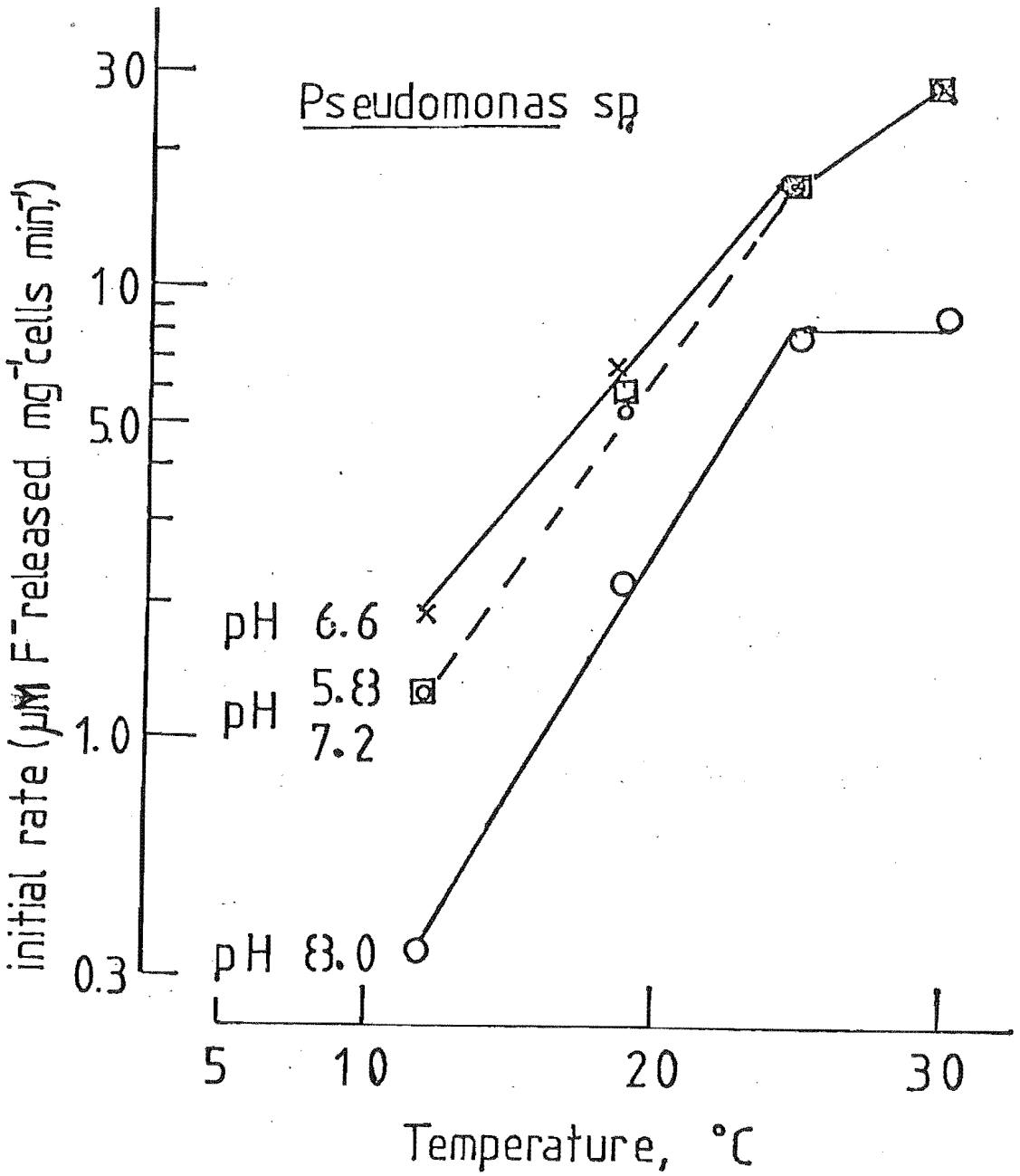
Defluorinating activity was assayed under conditions described in Figs 4.1 and 4.4a.
* exposed to acid pH for 15 min, assayed at pH 7.2.

BUFFERS

FLUOROHYDROLASE FROM:

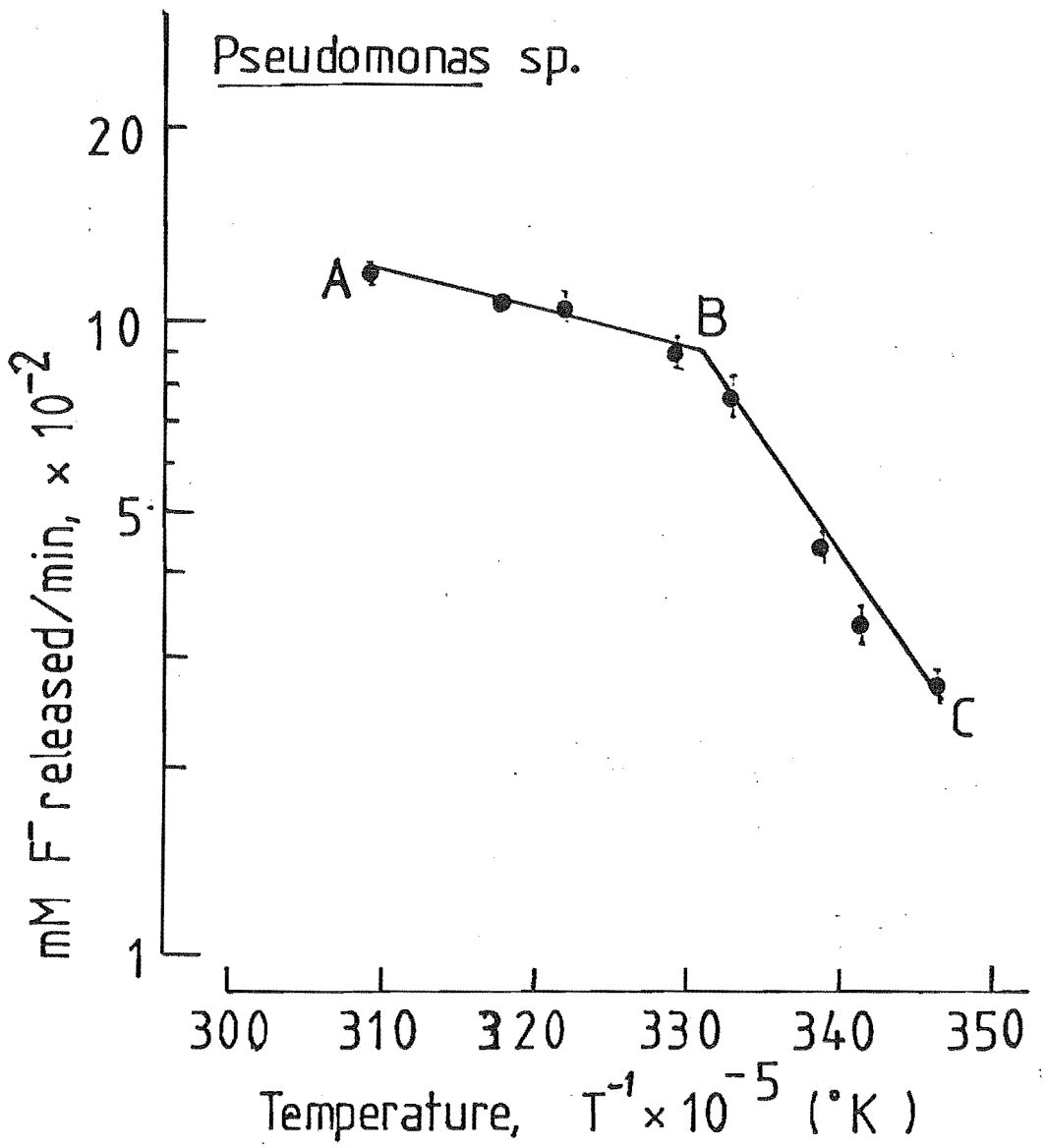
	Pseudomonas sp	*F. solani*
CITRATE/PHOSPHATE	■---■	●—●
PHOSPHATE	□---□	○—○
GLYCINE/NaOH	◪---◪	●—●

FIG 4.5A TRANSFORMED (SEMI-LOG) CURVES OF FIG 4.4A TO SHOW THE EFFECT OF TEMPERATURE: RESTING CELLS OF



curves of F^- released are logarithmically transformed to facilitate comparison of the effect of temperature at different pH on NaFa defluorination by resting cells of *Pseudomonas* sp.

FIG 4.5B: ARRHENIUS PLOT TO DETERMINE THE ENERGY OF ACTIVATION OF FLUOROHYDROLASE - NaFa DEFLUORINATION



The effect of temperature on the rate of NaFa catalysed by crude fluoorhydrolase preparation of *Pseudomonas* sp was assayed under standard conditions. The Arrhenius plot illustrates the transition from one level of activation to another at 32°C(B).

Equation (IV) $$E_A = \frac{d \ln k}{d T} = \frac{2.303\, d \log k}{d T^{-1}} = \frac{2.303\, RT^2 \log Q_{10}}{10}$$

where :

T = temperature in ^{o}K;

R = ideal gas constant, 8.3 joules per mole; and

k = rate of the reaction

The quantity, $\frac{\log k}{T^{-1}}$ is the slope of the line obtained from the Arrhenius plot. As depicted by Figure 4.5b, the plot showed a discontinuous slope, composed of two lines represented by the equations:

for Line AB: $\log V' = 6.335 - 0.010\ T^{-1}$

for Line BC: $\log V'' = 10.141 - 0.025\ T^{-1}$

The temperature at which the discontinuity in slope occurred was computed by simultaneous solving of the two equations above, and found to be $32^{o}C$. At this temperature the energy of activation (E_A) decreased from 47.7 kJ per mole for temperature range, 15-$32^{o}C$ to 19.7 kJ per mole for the range $32^{o}C$-$50^{o}C$.

4.3.5a Effect of metal activators and inhibitors

The sensitivity of fluorohydrolase from crude preparations of *Pseudomonas* sp. to metal ions: $Mg^{++}(MgSO_4)$, $Fe^{++}(FeSO_4)$, $Hg^{++}(HgCl_2)$, $Ag^{+}(AgCl)$, a metal chelating agent (NaN_3) and a thiol-specific agent (*p*-hydroxymercuribenzoate) was investigated. The enzyme was pre-incubated in the presence of possible activators or inhibitors as specified in Table 4.4, then assayed under standard conditions ($25^{o}C$, pH 7.2) and the initial rate of defluorination (first 10 mins) was used as the basis of comparison as before.

The rate of defluorination of NaFa by the enzyme was not affected by 1 mM Mg^{++} or Fe^{++}. However, at higher concentrations (10 mM) of either metal ion, the rate was reduced by approximately 10% (Fe^{++}) and 20% (Mg^{++}). Inhibition was not increased by prolonged pre-incubation up to 7 hours. Sodium azide, a general metal-chelator (Dixon and Webb, 1964) at 1.0 mM showed no inhibitory effect on enzyme activity. Heavy metal ions such as Ag^{+} amd Hg^{++} caused a significant reduction (52%) in the initial rate of defluorination when the enzyme was pre-incubated for 10 min with 0.1 mM Ag^{+} or Hg^{++} Simultaneous addition of Hg^{++} was less

Table 4.4: Effect of Thiol-specific agents Metal Chelators and Metal Ions

Preincubation Period	% Inhibition of Defluorinating Activity: (Based on F^- release)										
	Concentration of INHIBITORS/ACTIVATORS (mM)										
	*p*HMB		Hg^{++}		Ag^{+}		NaN_3	Mg^{++}		Fe^{++}	
	0.1	1.0	0.1	1.0	0.1	1.0	1.0	10.0	100	10	100
0.0 min	26		21		-	-	-	-	-	-	-
10 min	40	80	52	59	52	60	0.0	0.0	-	0.0	-
20 min	61	100	52	-	52	-	-	0.0	-	0.0	-
45 min	78	100	-	-	-	-	-	-	-	-	-
60 min	-	-	-	-	-	-	-	0.0	-	0.0	-
90 min	-	-	-	-	-	-	-	-	20	-	10
4 hours	-	-	-	-	-	-	-	-	20	-	10
7 hours	-	-	-	-	-	-	-	-	20	-	10

Reaction mixture (pH 7.2) contained 1.5 ml crude fluorohydrolase preparation of *Pseudomonas* sp. and the inhibitor or activator at the concentrations indicated, incubated at 25°C for 10 min.

*p*HMB = *p*-hydroxymercuribenzoate.

inhibitory causing a reduction of only 21% of initial rate of defluorination. The enzyme was also susceptible to inhibition by the thiol-specific agent, *p*-hydroxymercuribenzoate (*p*HMB). Simultaneous addition of the enzyme and the inhibitor (0.1 mM) caused a similar decline in the rate of defluorination as was observed with Hg^{++}. The similarity also extended to dependence on concentration of inhibitor and period of preincubation as shown in Table 4.4. Reduction in initial rate of defluorination became more marked with increase in inhibitor concentration and period of pre-incubation.

4.3.5b Effect of NaF: Product inhibition

Earlier studies on the introduction of NaF to culture flasks inoculated with soil inoculum (Chapter 2: 2.3.3.4) showed that defluorination was affected. Consequently attempts were made to study the type of inhibitory mechanism using intact resting cells and crude enzyme preparations of *Pseudomonas* sp. The inhibitor constant (K_i) with whole cells was determined by a Dixon plot (Figure 4.6a) to be 20 mM, about twice that determined by Linweaver-Burke Plot (Figure 4.6b) for the soluble enzyme preparation, thus showing that the isolated enzyme was more susceptible to NaF inhibition than whole bacterial cells. Both plots showed F^- to be a mixed type inhibitor affecting both K_m and V_{max}.

4.3.6 Substrate Specificity

A range of fluoro-organic compounds and halogenated aliphatic acids were tested as substrates for resting cells and crude enzyme preparations of *Pseudomonas* sp. and *F. solani*. These compounds were dissolved in 20 mM phosphate buffer, pH 7.2 and made up to the same concentration as NaFa (20 mM). Fluoroacetanilide and naphthyl methyl fluoroacetamide (NMFA) were only sparingly soluble and therefore used as suspensions. All compounds were assayed under the same conditions (incubated at 25°C for 30 mins).

The relative activities of the cells and crude enzymes on various substrates is shown in Table 4.5 where it may be seen that all cell-free preparations were more active on monohalogenated acetates with iodoacetate as an exception, compared to other fluoro-organic compounds as substrates. Whilst cell-free

Fig 4.6a Dixon plot to determine K_i of F^-: resting cells of Pseudomonas sp.

Inhibition by fluoride ions - Dixon type plot to estimate K_i value at resting cell level. Defluorinating activity was assayed under pH 7.2, otherwise under conditions described in Fig 4.1.

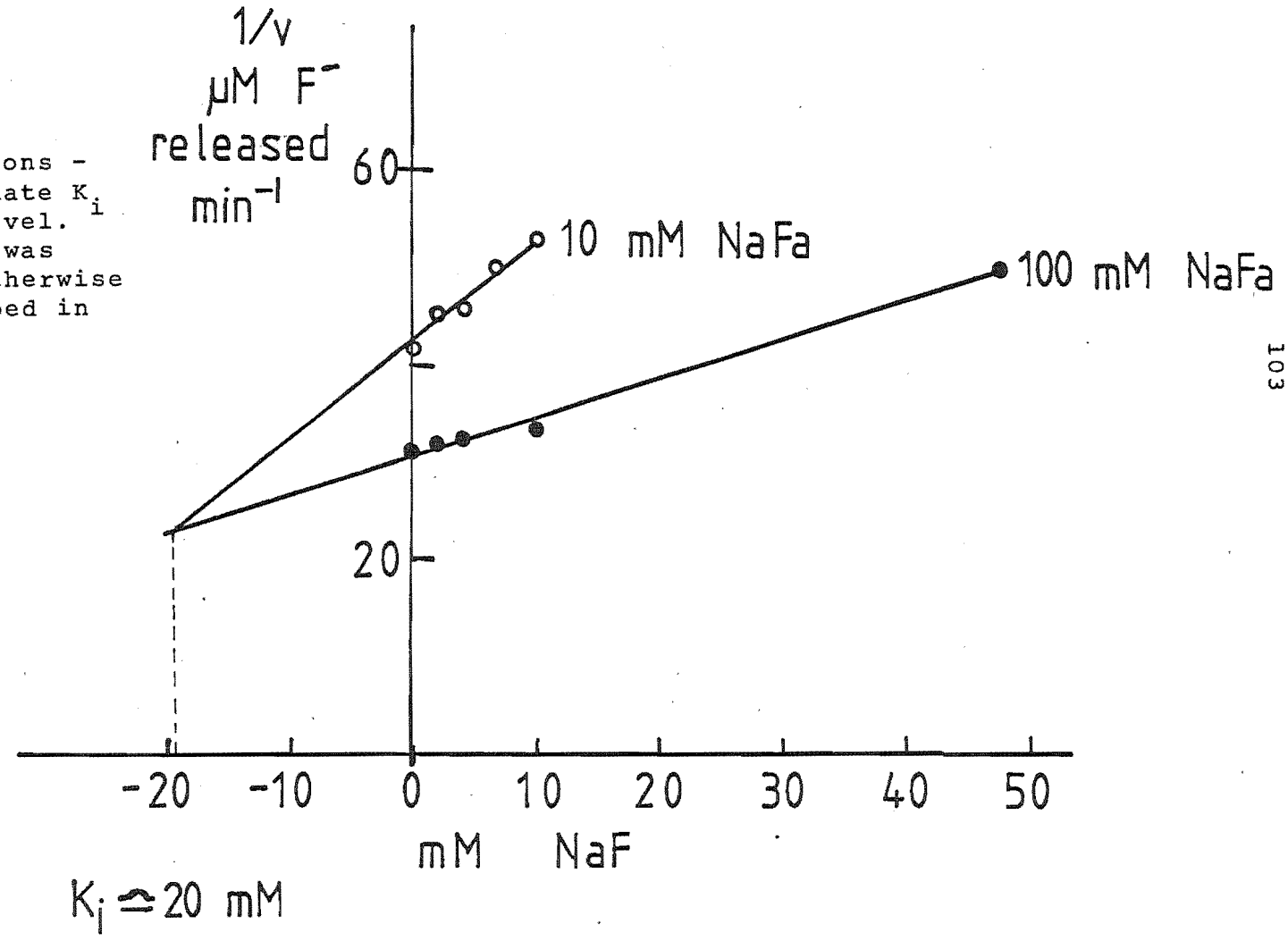
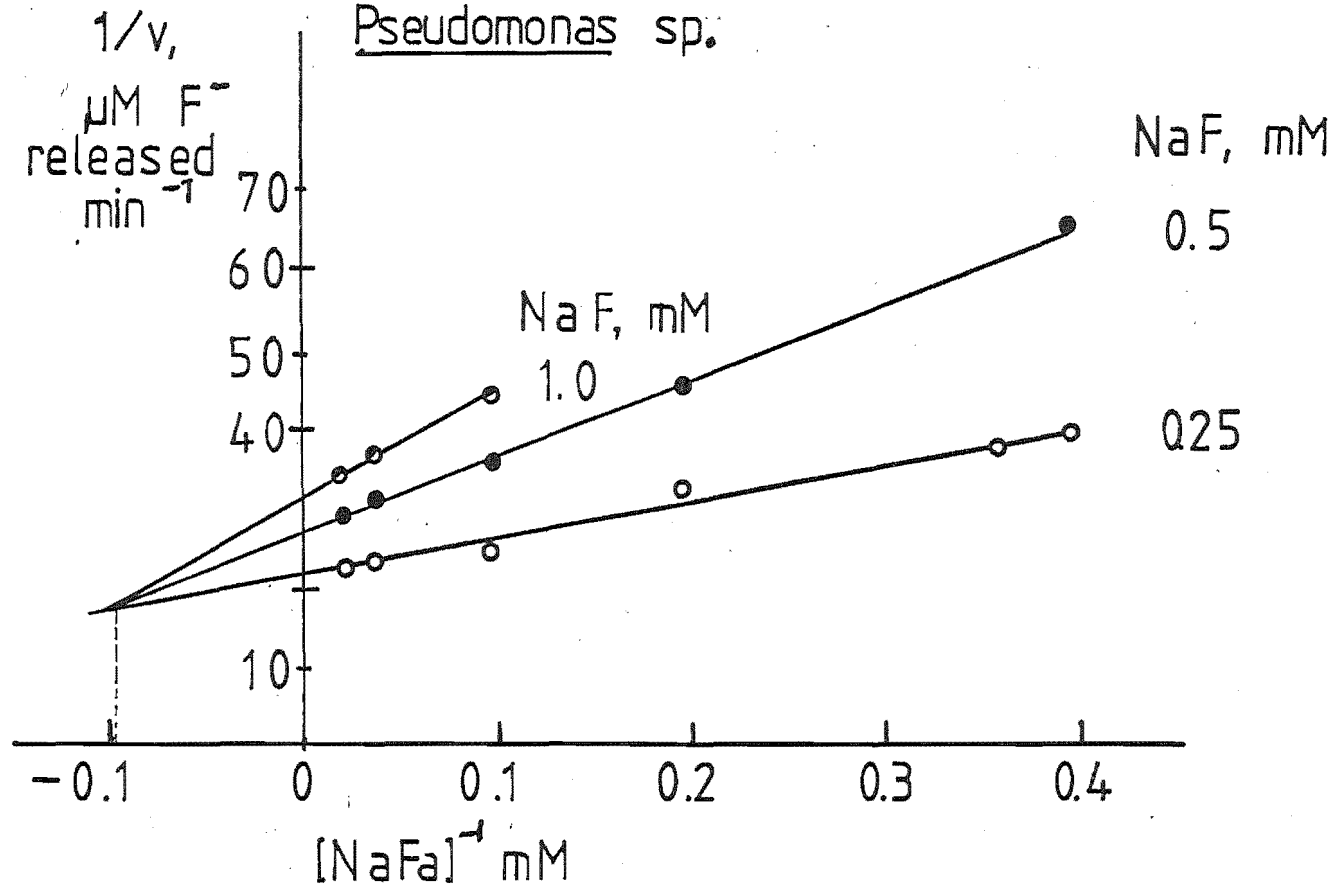


Fig 4.6b Lineweaver — Burke plot to determine K_i of F^- : fluorohydrolase



$K_i \simeq 9.1$ mM : Assay conditions as described in Fig 4.1

Table 4.5: Substrate Specificity

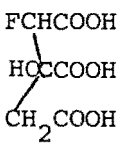
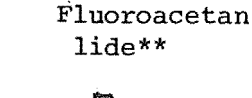
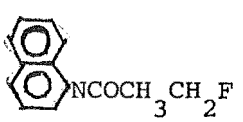
Substrate	Product assayed	mM substrate dehalogenated relative dehalogenating activity (%)			
		Pseudomonas sp.			*F. solani*
		whole cells	freeze-dried	Fluorohydrolase	Fluorohydrolase
Fluoroacetate FCH_2COO^-	F^-	2.2 (40.0)	4.4 (80.0)	5.5 (100)	3.2 (100)
Fluoroacetamide FCH_2CONH_2	F^-	1.5 (27.3)	1.8 (32.8)	2.3 (49.3)	1.0 (31.3)
Difluoroacetate F_2CHCOO^-	F^-	0.14 (2.5)	0.68 (12.4)	0.75 (13.6)	
Difluoroacetamide $F_2CHCONH_2$	F^-			0.0	
Trifluoroacetate F_3CCOO^-	F^-	0.13 (2.4)	0.40 (7.3)	0.66 (12.0)	0.16 (5.2)
Trifluorochloropropionate $FClHCCF_2COO$	F^-			0.0	
Tetrafluoropropionate $F_3CFCHCOO^-$	F^-			0.0	
$F_2CHCF_2COO^-$				0.0	
Fluorocitrate 	F^-			0.0*	
Fluoroethanol FCH_2CH_2OH	F^-	0.22 (4.0)	0.60 (10.9)	0.69 (12.6)	
Fluorobutyric ethylester $C_2H_5CHFCOO-CH_2CH_3$	F^-	0.27	0.52	0.76	

Table 4.5 contd

Substrate	Product assayed	mM substrate dehalogenated relative dehalogenating activity (%)			
		Pseudomonas sp.			*F. solani*
		whole cells	freeze-dried	fluorohydrolase	fluorohydrolase
Fluorocinnamic 	F^-	0.12 (2.2)	0.4 (7.3)	0.62 (11.3)	
Fluoroacetanilide** 	F^-	0.16 (2.9)	0.46 (8.5)	0.68 (12.4)	0.18 (5.4)
Naphthyl-methyl-fluoroacetamide 	F^-	0.22 (4.1)	0.38 (6.9)	0.68 (12.4)	0.18 (5.4)
Chloroacetate $ClCH_2COO^-$	glycolate	0.0*		1.37* (25.0)	0.8* (25.0)
Bromacetate $BrCH_2COO^-$	glycolate	0.0*	0.92* (16.7)	2.0* (36.4)	
Iodoacetate ICH_2COO^-	glycolate	0.0*	0.2* (3.6)	0.2* (3.6)	

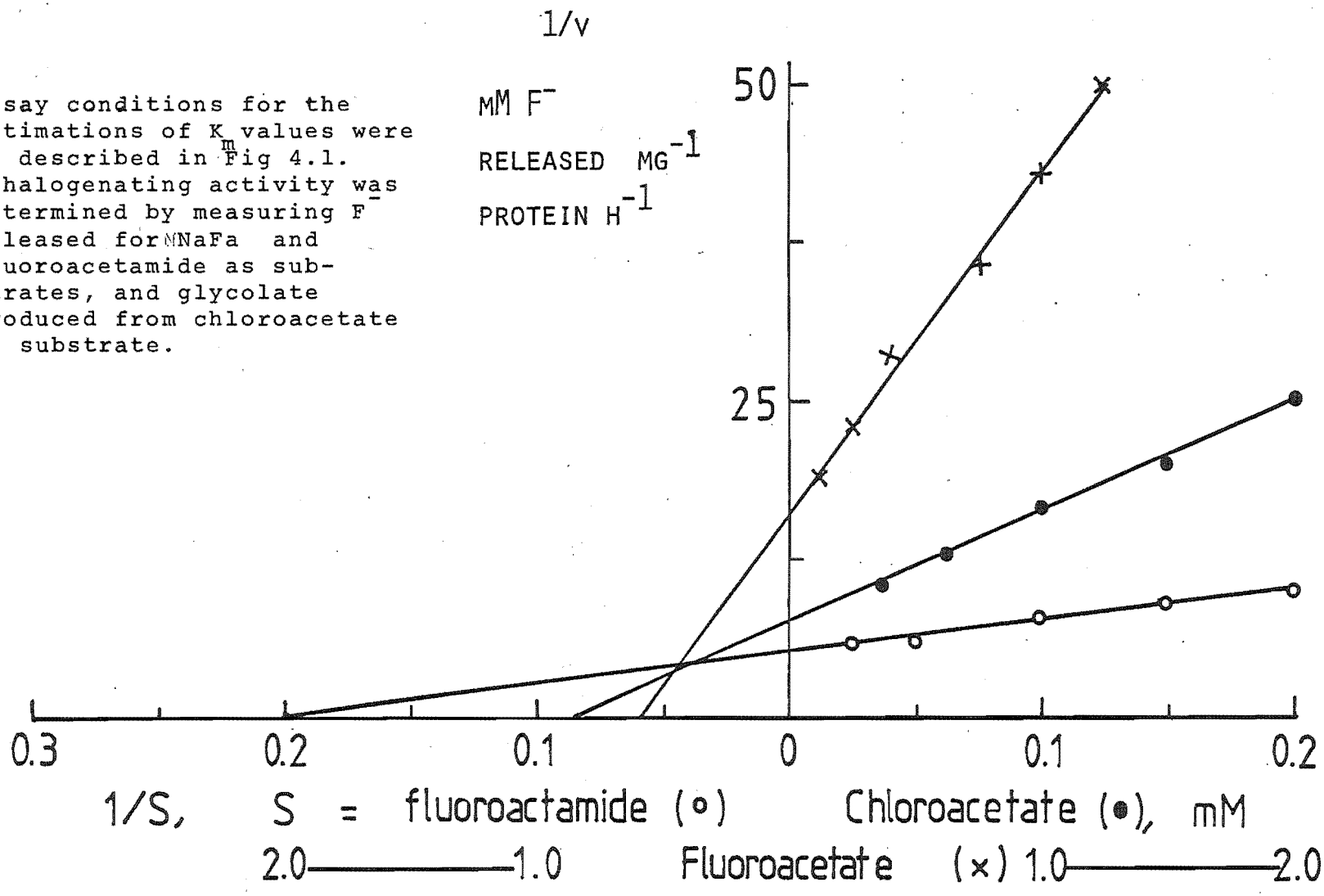
Conditions of assay: Reaction mixture contained 10 mM substrate and cell or crude enzyme preparation (pH 7.2), incubated at 25°C for 30 min, then F^- or glycolate measured. Results are averages of triplicates.

* Values corrected for spontaneous dehalogenation.

** Substrate is sparingly soluble - used as suspension.

preparations could dehalogenate chloro- and bromoacetate, intact bacterial cells showed no detectable activity on these substrates which underwent spontaneous dehalogenation (data entered into Table 4.5 had been corrected for spontaneous dehalogenation). Bromoacetate was dehalogenated by crude enzyme preparations of *Pseudomonas* sp. to a greater extent than chloroacetate. Acetates which have more than one fluorine-substitution were attacked by both resting cells and enzyme preparations to only a limited extent. Fluoropropionates and fluorochloropropionate were not dehalogenated when tested with crude enzyme preparation of *Pseudomonas* sp. Although fluoroethanol and α-fluorobutyric ethyl ester have the fluorine atom substituted at a similar carbon atom (C_2) as fluoroacetate, they were only poorly attacked by resting cells and enzyme preparations. Little activity was observed on di- or trifluoroacetate; and whilst fluoroacetamide was attacked there was little or no activity against NMFA or difluoroacetamide. Aromatic fluoro-organic compounds were also attacked to a limited extent. Fluorocitrate spontaneously dehalogenated and was unaffected by the enzyme preparation from *Pseudomonas* sp. These results show that both resting cells and cell-free extracts of *Pseudomonas* sp. and *F. solani* were highly specific for fluoroacetate and its close analogues; fluoroacetamide, bromo- and chloroacetate, but not iodoacetate. The specificity of enzyme preparations of *F. solani* and *Pseudomonas* sp. was studied further by determining the K_m (and V_{max}) values on the above mentioned analogues. K_m was determined using Lineweaver-Burke plots as shown in Figure 4.7, and given in Table 4.6. *F. solani* extracts showed higher K_m values than the *Pseudomonas* enzyme indicating that the *F. solani* enzyme has a lower affinity for fluoroacetate. K_m values for fluoroacetamide and chloroacetate obtained with *Pseudomonas* extract were about 3 times lower than those obtained with *F. solani* extract which suggests that *Pseudomonas* enzyme was relatively less specific.

FIG: 4.7 LINEWEAVER-BURKE PLOTS TO DETERMIME K_M OF FLUOROACETATE, FLUOROACETAMIDE AND CHLOROACETATE



Assay conditions for the estimations of K_m values were as described in Fig 4.1. Dehalogenating activity was determined by measuring F^- released for NaFa and fluoroacetamide as substrates, and glycolate produced from chloroacetate as substrate.

Table 4.6: Comparison of *Pseudomonas* sp. and *F. solani* Fluorohydrolase Activity on Different Substrates

Substrate	K_m (mM)		V_{max} F^- released (mM) $mg^{-1} hr^{-1}$	
	Pseudomonas sp.	*F. solani*	*Pseudomonas* sp.	*F. solani*
Fluoroacetate*	1.97±0.09	2.07±0.33	16.88	8.02
Fluoroacetamide*	4.16±0.45	15.64±1.51	5.5	20.44
Chloroacetate**	10.12±0.05	21.11±0.18	8.26	8.04

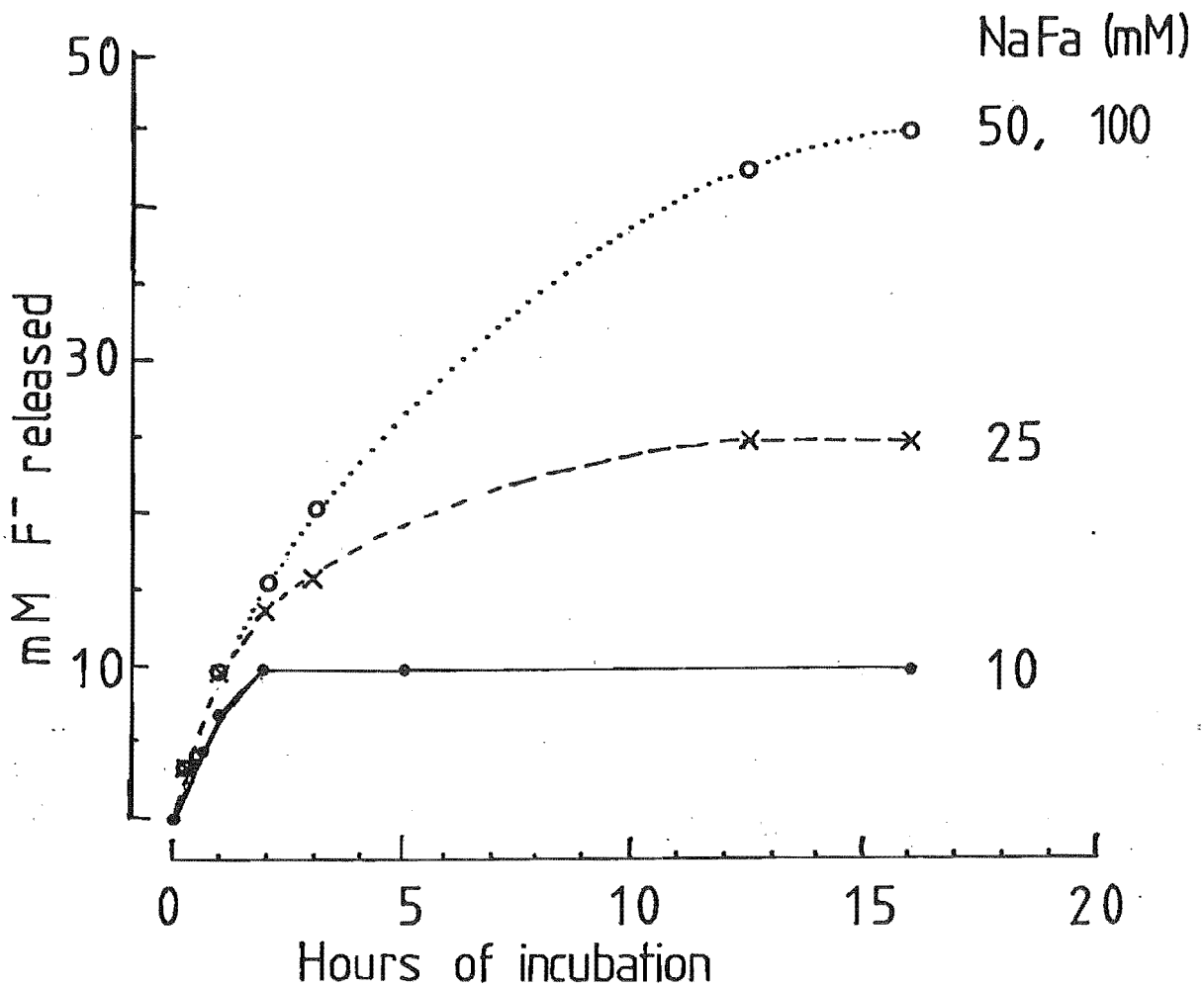
Activity followed by F^- (*) or glycolate determination (**). Activity was assayed under conditions described in Fig. 4.7.

The activity of resting cells against all compounds tested as substrates was lower than that exhibited by freeze-dried cells (partially broken) and cell-free extracts of *Pseudomonas* sp. although all preparations were made with equal concentration of cells. This may suggest that the cell membrane may form a barrier reducing accessibility of the substrates to the enzymes and thereby apparently reduced activity was observed.

4.3.7 Effect of Substrate and Enzyme Concentration on the Rate of Defluorination

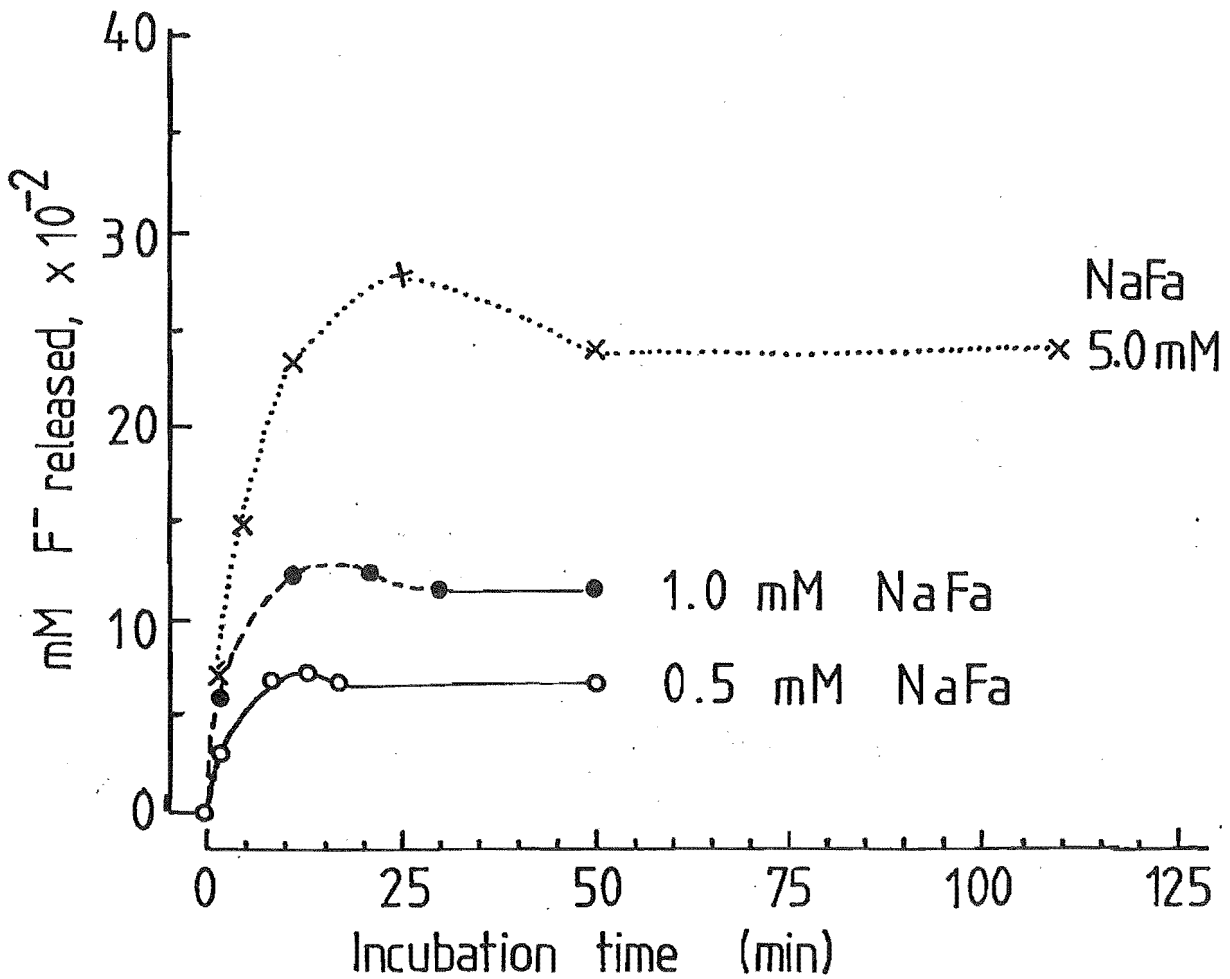
Time course studies of the effect of increasing substrate and enzyme concentrations were made with a view to assessing the potential of the enzyme for the bioassay of NaFa. For these studies crude enzyme preparations of *Pseudomonas* sp. were used and some experiments were repeated using resting cells. The rates of defluorination were determined by measuring F^- released into the reaction mixture incubated at 25°C at 1-2 minute intervals for 10 minutes. Longer incubation periods were used with resting cells and in time course studies. Progress curves of NaFa defluorination at constant enzyme and cell concentrations with various concentrations of NaFa are shown in Figures 4.8 and 4.9. The rates of cellular and enzymatic defluorination of NaFa decreased with time, more marked with the enzyme preparation, until an equilibrium was reached. Although the rate of defluorination by resting cells also decreased with time an equilibrium point was not observed and defluorination continued until all the NaFa was defluorinated. At each enzyme level, the initial rates of defluorination increased with increasing concentration of NaFa as shown in Figures 4.10a and 4.10b. The factor of

Fig 4.8 Time course study of defluorination of NaFa : resting cells of Pseudomonas sp.



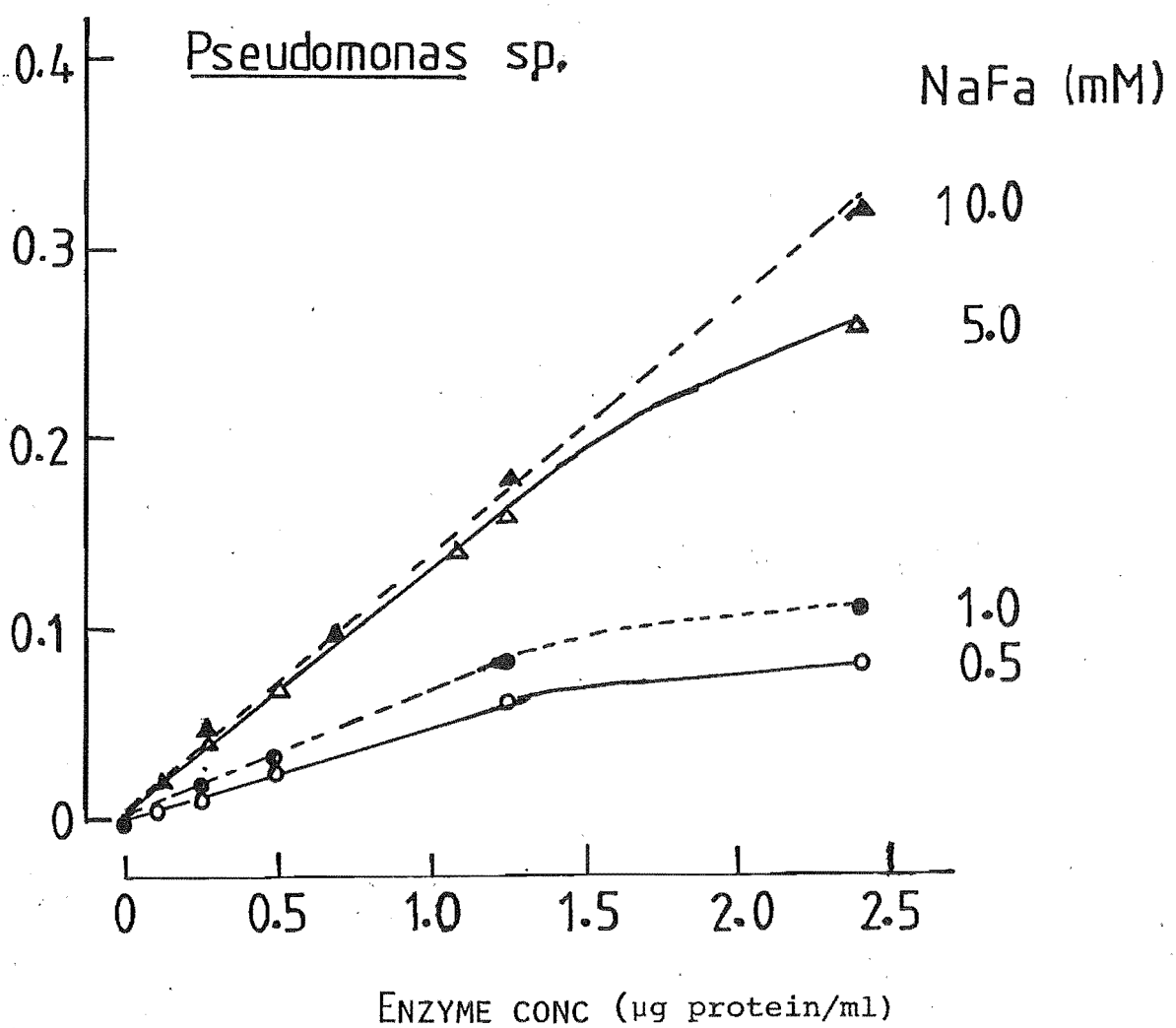
Kinetic studies with resting cells of *Pseudomonas* sp - reaction mixture contained 1.5 ml cell suspension and 1.5 ml NaFa at various concentrations, incubated at 25°C, and F^- released measured. Results expressed are means of triplicates.

Fig 4.9 Time course study of defluorination of NaFa : fluorohydrolase of Pseudomonas sp.



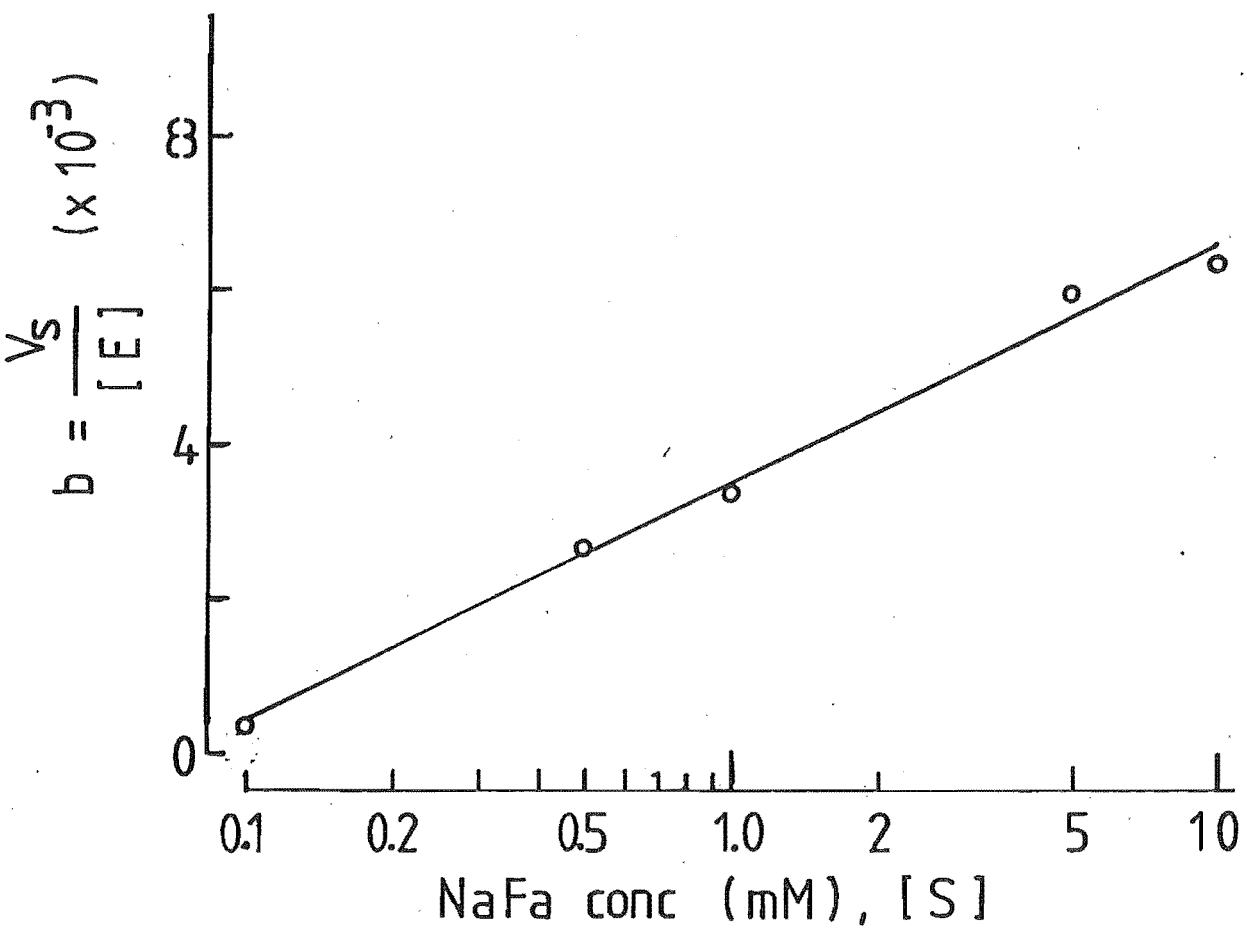
Kinetic studies with crude fluorohydrolase preparation of *Pseudomonas* sp. - Assay conditions were as described in Fig. 4.8. Results are means of triplicates.

FIG 4.10A EFFECT OF ENZYME AND SUBSTRATE CONCENTRATION ON THE INITIAL VELOCITY OF DEFLUORINATION: FLUOROHYDROLASE



Reaction mixtures contained 1.5 ml enzyme solution and NaFa solution at different concentrations, incubated at 25°C, the rate of F^- released was followed over a period of 10-15 min. Results are averages of triplicates.

Fig 4.10b Relationship between enzyme and substrate concentration & the rate of defluorination



$$\frac{V_S}{[E]} = a + k \log[S]$$

$$= -5.9 + (3.1 \pm 0.1) \log [S]$$

V_S : Initial velocity of defluorination

[E]: Enzyme concentration

[S]: Substrate concentration

increment or enhancement on the rate of defluorination as a result of increasing enzyme concentration at each NaFa concentration was calculated by plotting the initial rates of defluorination at each NaFa concentration against enzyme concentration as shown in Figure 4.10a. The line is represented by the equation (Va):

Equation (Va): $$\frac{V_s}{[E]} = b$$

where 'Vs' denotes initial velocity of defluorination of NaFa concentration = [S] and at enzyme concentration = [E]; and 'b' is the factor by which Vs increases with increasing [E]. Values of 'b' obtained for the various combinations of [E] and [S] were plotted against [S] as shown in Figure 4.10b, represented by equation (Vb):

Equation (Vb) $$\frac{V_s}{[E]} = b = a + k \log [S]$$

where $1.0 \leq [S] \leq 10$ mM;

$k = \frac{V_s}{[E][S]}$, slope of the line shown in Fig. 4.10; and

$a = \frac{V_s}{[E]}$ when $\log [S] = 0$

'k' may be termed as substrate coefficient of 'b' as it measures the change in 'b' resulting from a change in NaFa concentration. This equation is useful in that it relates the rate of defluorination to enzyme and NaFa concentrations and Figure 4.10b could be used as a standard curve for determining NaFa concentration.

4.4. DISCUSSION

4.4.1. Characteristics of Defluorinating Enzymes of *Pseudomonas* sp. and *F. solani*.

Attempts to obtain sufficient quantities of pure defluorinating enzyme (fluorohydrolase) from *Pseudomonas* sp. and *F.solani* were hampered by several factors. One of these was the low yield of protein - 3.5 mg protein from 10 mg (dry weight) bacterial cells, and 6.5 mg protein from 20 g (wet weight) fungal mycelium. The recovery of the enzyme from subsequent purification processes

was poor - less than 10% in a highly diluted form after gel filtration or ion exchange chromatography for only 4 to 8-fold increase in specific activity. Another factor was the local unavailability of adequate facilities for large scale culture of organisms (fermenter) and harvest of bacterial cells (continuous-flow centifuge). Furthermore NaFa was not a good carbon source for growth. Despite presence of an optimal balance of glucose (to enhance growth) and NaFa (to induce defluorinating activity) to the growth medium, the final yield of the enzymes required, compared to other proteins present, was still insufficient to overcome the losses and considerable dilutions that occurred during purification. For these reasons, crude or cell-free enzyme preparations were used for most of the characterisation and biochemical studies.

The molecular weight of the defluorinating enzyme of *Pseudomonas* sp. and *F. solani* (thereafter referred to as bacterial or fungal fluorohydrolase or simply enzymes) as estimated by means of a pre-calibrated Sephadex G-150 column were found to be similar, around 45,000-68,000. This similarity extended to their response to pH with optima at pH 8.0. The pH- response curves of both enzymes were more or less parallel, having common points of inflexion. This suggests that both enzymes possessed common ionisable groups at similar positions along the active or binding site of their polypeptide chains. Both enzymes were relatively more active at alkaline pH, with defluorinating activity increasing as the pH was raised to 8.0 and decreasing as the pH was raised beyond 8.0. This suggests that the defluorinating activity of both enzymes may be regulated more by the anionic than the cationic groups of the enzymes or, alternatively, by the availability of OH^- or NaFa in a dissociated form (pK_a fluoroacetic acid = 3.81). Acid inactivation of the enzyme was reversible, indicating that at low pH, the enzyme had not been denatured but that the reduced activity was the effect of pH on NaFa, availability of OH^-, or ionic groups of enzymes as suggested above.

Some dissimilarity of the two defluorinating enzymes was observed with respect to their thermal stability - the fungal

enzyme preparation was more thermo-labile. Whilst the bacterial enzyme remained active after incubation at 55°C for 60 minutes and its specific activity was enhanced, the fungal enzyme was completely inactivated. The enhanced activity observed of the heat-treated bacterial preparation (assayed at 25°C) may be the result of the removal of some inhibitors present by heat precipitation. Thermal inactivation of both enzymes was irreversible.

Earlier studies on fluorohydrolases from other *Pseudomonas* species by other workers (Goldman, 1965; Tonomura *et. al.*, 1966) showed that the optimal pH was within pH 8.0-9.3 despite the different sources from which the enzyme was extracted. However differences in thermal stability were reported. The fluorohydrolase studied by Goldman (1965) lost 80% of its defluorinating activity (pH 6.9) when incubated at 47°C for 50 minutes whereas the bacterial fluorohydrolase presently studied showed enhanced activity after being heated at 55°C for 60 minutes. The fluorohydrolase studied by Tonomura *et. al.* (1966) was more stable at 5°C (pH 9.1) than when frozen whereas the enzyme from *Pseudomonas* sp. and *F. solani* presently studied was less stable at 6°C (pH 7.2) than when frozen. These differences may not be due to differences in stability of the enzyme structure but rather a consequence of differences in purity of the enzyme preparations, some of which may have contained proteolytic enzymes.

4.4.2. Metal Requirements and Inhibitors

Both Mg^{++} and Fe^{++} are reported to be activators of many enzymes such as synthetases, kinases, decarboxylases and phosphatases. Their effect on defluorinating activity of crude enzyme preparations of *Pseudomonas* sp. was studied and shown to be without activating or inhibitory effects. A commonly used metal-chelator, NaN_3, was also tested and shown to be without effect on defluorinating activity; these observations suggest that the enzyme had no requirements for specific metal activators.

However, the defluorinating enzyme was susceptible to inhibition by a thiol-specific agent (*p*-hydroxymercuribenzoate) suggesting that thiol groups are involved at the active site of the enzyme. Moreover the enzyme was also inhibited by heavy

metals such as Ag^{+} and Hg^{++} which are known to inactivate enzymes by alkylating their -SH groups, thus confirming further the importance of thiol groups at the active site. The enzyme was also inhibited by F^{-}, one of the products of NaFa-breakdown, F^{-} was a mixed type competitive inhibitor affecting both V_{max} and K_{m}, probably affecting the enzyme via end-product inhibition. Fluorohydrolases studied by earlier workers (Goldman, 1965; Tonomura *et. al.*, 1966) was also reported to be sensitive to thiol-specific agents.

4.4.3 Substrate Specificity

a. Whole cells vs enzyme preparation - The accessibility of substrates as regulated by a membrane barrier was investigated by comparing the relative rates of dehalogenation of these substrates by whole cells, freeze-dried (partially broken) cells and cell-free extracts of *Pseudomonas* sp. as shown in Table 4.5. For all substrates the dehalogenating activity increased in the order indicated

whole cells < freeze-dried cells < crude fluorohydrolase preparation

Glycolate was not detected in the incubation mixture of whole cells and iodo-, bromo-, or chloroacetate. This may suggest that the cells had either oxidised the glycolate produced or that these halogenated aliphatic acids were not attacked by the cells due to the presence of a membrane barrier. However the carbon-halide bonds of these substrates were cleaved by freeze-dried cell and fluorohydrolase preparations. Thus the results show that the low activity shown by intact cells against the range of substrates tested could be explained by the presence of a membrane barrier which regulates the entry or accessibility of the substrates to the enzyme.

b. Specificity of fluorohydrolase of *Pseudomonas* sp. and *F. solani*

The substrate specificity of fluorohydrolase of *Pseudomonas* sp. and *F. solani* was tested for a range of fluoro-organic compounds and chemical analogues of NaFa as indicated in Table 4.5. It was found that the fungal enzyme has a lower affinity for fluoroacetate compared to the bacterial enzyme.

Both enzymes were able to cleave the carbon-halide bond of halogenated analogues of fluoroacetate in order of decreasing activity:

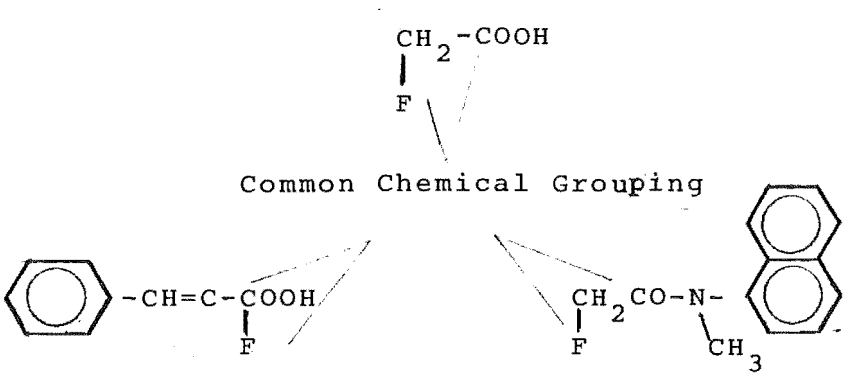
$F\text{-}CH_2COOH > F\text{-}CH_2\text{-}CONH_2 > Br\text{-}CH_2\text{-}COOH > Cl\text{-}CH_2\text{-}COOH > I\text{-}CH_2\text{-}COOH$

The bacterial enzyme was less specific than the fungal enzyme, a conclusion derived from the relative activities on FCH_2COOH, FCH_2CONH_2 and $ClCH_2COOH$.

c. Specificity for Monohalogenated Acetates vs Others

Results based on studies with the defluorinating enzyme from *Pseudomonas* sp. show that the enzyme had a higher specificity for monohalogenated acetates, whilst little or no activity was observed on other aliphatic fluoro-organic compounds with one or more fluorine-substitutions and on aromatic fluoro-organic compounds. There are several factors which seem to determine whether F-compounds can be attacked by the enzyme for example, solubility, and hence availability, chemical configuration of the substrates and the position of substituents. The low solubility of *p*-fluoroacetanilide and N-naphthyl-N methylfluoroacetamide (NMFA) may account for their apparent resistance to attack by the defluorinating enzyme. On the other hand the recalcitrance of these substrates may be real since in both α-fluorocinnamic acid and NMFA the

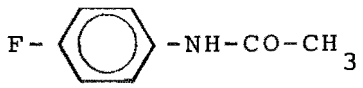
Fluoroacetic acid



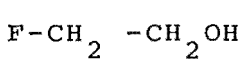
α-fluorocinnamic acid N-naphthyl-N-methylfluoroacetamide

fluorine is substituted at the same carbon position as fluoroacetate. The resistance of these substrates could possibly be the result of steric hindrance imposed by the aromatic substituents. Both steric hindrance and position of the fluorine substitution may account for the resistance

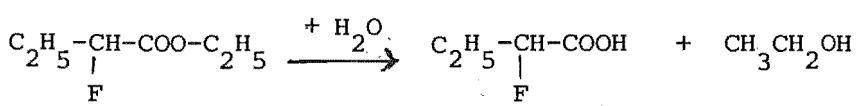
of *p*-fluoroacetanilide to defluorination.



However, steric hindrance would not be expected to affect fluoroethanol which differs from fluoroacetate only in lacking a carboxyl group. This may suggest that



a carboxyl group may be necessary for binding to the defluorinating enzyme. However fluoroacetamide, which has no carboxyl group, was shown to be readily defluorinated by the enzyme, almost certainly due to easy hydrolysis of the amide to yield fluoroacetate. Ethanol has been reported to be inhibitory to dechlorinating activity (Bray *et. al.*, 1952), probably by denaturing the protein, but protein precipitation was not detected in fluoroethanol mixtures with the defluorinating enzyme. Likewise the low activity on α-fluorobutyric ethyl ester may be as if it hydrolysed to form fluorobutyric acid and ethanol.



fluorobutyric ethyl ester — α-fluorobutyric acid — ethanol

The alkyl group attached to the α-carbon of fluorobutyric acid may shield the C-F bond or prevent binding of the molecule with the enzyme, suggesting that the nature of other substitutions at the α-carbon to which the fluorine atom is attached may determine whether the substrates would be attacked. Other examples in which secondary substitutions conferred resistance of the substrates to attack by the defluorinating enzymes were difluoroacetate, trifluoroacetate and tetrafluoropropionic acids. Additional fluorine atoms, which are slightly larger than hydrogen atoms, would further increase the size of the substrate molecule with the consequence that the C-F bond was made less accessible to the enzyme and therefore lower activity on these substrates was observed.

4.4.3d Relative specificity for carbon-halide bonds

The defluorinating enzyme studied showed high specificity for

monohalogenated acetates. An attempt was made to examine the relative specificity of the defluorinating enzyme for C-F bonds, other carbon carbon-halide (C-X) bonds and the acetate radical of monohalogenated acetates. It appears from the relatively high activity on fluoroacetate and fluoroacetamide that the enzyme was specific for C-F bonds when compared to chloro- or bromoacetate as substrates. However as discussed above, the specificity for the C-F bond is conditional upon other substituents of the α-carbon atom. Compared with di- and trifluoroacetates as substrates, the activity of the enzyme on chloro- and bromocetate was relatively higher. It is thus deduced that specificity for C-F bonds is higher than for other C-X bonds but only in the presence of acetate or acetamide, thus:

$X\text{-}CH_2\text{-}COO^-$, or $X\text{-}CH_2\text{-}CONH_2$ where X = F, Cl, Br.

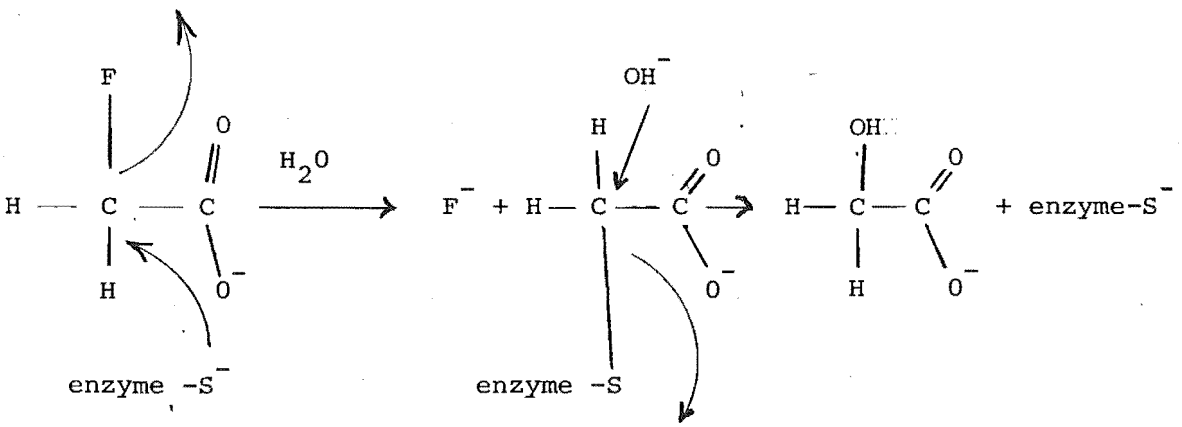
A notable exception was that the enzyme showed no activity on iodoacetate, the carbon-iodide bond of which is weaker than C-Br and C-Cl bonds of bromo- and chloroacetate (Refer Table 1.1). Mention should be made here that in solutions iodo-, bromo and chloroacetates were found to dehalogenate spontaneously and this was allowed for when comparing relative substrate specificities. On the basis of bond strength, iodoacetate should be more easily dehalogenated than bromoacetate and chloroacetate. The low activity of the defluorinating enzyme on iodoacetate could be attributed to the thiol-alkylating property of iodoacetate (Dixon and Webb, 1964). The importance of -SH groups at the active site of the enzyme was strongly implicated from the present inhibitor studies.

The specificity of the defluorinating enzyme of *Pseudomonas* sp. was broader than that studied by Goldman (1965), the K_m for chloroacetate as found in present studies was 6 times that of NaFa, while that studied by Goldman (1965) showed a 10-fold difference. Both preparations showed similar high specificity for monohalogenated acetates.

4.4.4. Mechanism of NaFa Defluorination

The mechanism of enzymatic defluorination of fluoroacetate

has been studied by Goldman & Milne (1966), using labelled $H_2^{18}O$ and mass spectrometric analysis of the product, glycolate. Goldman *et. al.*, (1968) postulated a nucleophilic attack by the thiol group of fluorohydrolase followed by hydroxyl displacement as shown below:



Several findings from the present studies are in accord with this suggested mechanism. Inhibitor studies showed that -SH groups were strongly implicated and that defluorination was reduced under high $[H^+]$ conditions but was relatively unaffected by high $[OH^-]$ concentration.

Based on studies of the effect of temperature on kinetics of enzymatic defluorination of NaFa, activation energy involved in defluorination of NaFa was estimated to be 47.7 kJ per mole within the temperature range, 15 - 32°C and 19.7 kJ per mole between 32 - 50°C (Cf: C-F bond energy of fluoroacetic acid is 447.9 kJ per mole). At present there is insufficient information on the phenomenon of a transition from one activation energy to another at a particular temperature as observed in some enzyme-catalysed reactions. Another example of this phenomenon which has been studied more fully is the hydration of fumarate catalysed by fumarate hydratase and discussed by Dixon & Webb (1964). Several explanations have been offered, one being that the enzyme molecule is dissociated at higher temperature into smaller active sub-units with lower activation energies. Another theory suggests that two successive reactions are involved, each with its own temperature coefficient.

4.4.5 Influence of pH and Temperature

Comparisons were made of the effect of pH and temperature

on NaFa defluorination as catalysed by growing organism or resting cells and cell-free enzyme preparations. NaFa defluorination by growing mycelium of *F. solani* was maximal at pH 5.8 while cell-free enzyme preparation showed maximal activity at pH 7.8-8.0. The ability of fungi to adjust their own internal pH (Cochrane, 1958; Turner, 1975) may explain in part the differences observed. However the presence of a cell membrane as a barrier to entry of fluoroacetate and the effect of pH on the state of ionisation of the substrate may be the primary factor contributing to the difference in pH optimum observed. Small organic acids enter intact cells both by diffusion and active transport mechanism and the relative rate of entry by either mechanism depends on the state of ionisation of the substrate molecules and hence pH (Rothstein, 1965). The pK_a or dissociation constant of fluoroacetic acid is 3.81, and a change from pH 6.0 to 4.0 would increase the concentration of undissociated fluoroacetic acid molecules 60-fold (Black & Hutchens, 1948). Hence diffusion of fluoroacetic acid molecules into intact mycelium or cells would be favoured at lower pH values, and higher rates of defluorination were found at pH 5.8 and lowest at pH 7.8 for intact growing mycelium of *F. solani*. Further evidence on the crucial role of diffusion and hence availability of the substrate in determining the degree of defluorination catalysed by the biological agent was the 3-fold difference in temperature coefficients (Q_{10}) of defluorination by resting cells (7.3) and by cell-free enzyme preparation (1.9).

However, the rate of NaFa defluorination by intact (resting or growing) cells is not totally regulated by the rate of diffusion, though it may be the main factor as evidenced from the higher sensitivity of defluorination to pH compared to growth (Chapter 3) of *F. solani*. At higher pH, active uptake of fluoroacetate ions may become increasingly important but pH also influences uptake of other essential ions such as NH_4^+ and $PO_4^{\equiv}$, (Rothstein, 1965; Berry, 1975). At pH's above 6.5, uptake of $PO_4^{\equiv}$ rapidly declines while NH_4^+ uptake is favoured, consequently NaFa defluorination and growth of the organism may be limited at either acid or alkaline pH.

Studies of NaFa defluorination at cellular and enzymatic levels show that factors as pH or temperature affect the

activity of the biological agents differently. The basis of the difference lies chiefly on the presence or absence of a cell membrane barrier which introduces other factors such as diffusion or ionisation so that varying one environmental parameter leads to an interplay of a more complex multiple effect of this variable. The complexity or multiplicity of the effects increases from studies at enzymatic level to studies at cellular level and at soil level where numerous other organisms would interact.

4.4.6 Potential of Fluorohydrolase and Bacterial Cells as Tools for the Bioassay of NaFa

The relatively high specificity of fluorohydrolase or cells of *Pseudomonas* sp. suggested a potential use for these preparations for the quantitative determination of fluoroacetate.

Cellular method- As shown in Section 4.3.7, bacterial cells would completely defluorinate NaFa when incubated with it. Thus a sample with an unknown concentration of NaFa could be incubated with NaFa-adapted cells and the amount of F^- released measured with a F^- specific electrode. The period of incubation required would depend on the concentration of NaFa and the density of the cell suspension until a constant F^- concentration and NaFa level could be deduced. Thus the method is based on total F^- released into the incubation mixture.

Enzymatic method - Unlike the experiments with whole cells, the reaction catalysed by enzymes cannot proceed to completion unless the products are constantly removed. Therefore, instead of measuring total F^- released, the initial rate of defluorination was measured to overcome this obstacle. The enzymatic method was based on the principle of proportionality of the initial rate of defluorination (V_s) substrate ([S]) and enzyme concentration ([E]), as expressed by the equation (Vb):

Equation (Vb)
$$\frac{V_s}{[E]} = b = a + k \log [S]$$

where 'a' is the quantity, $\frac{V_s}{[E]}$ at log [S] = 0.0 and 'k' is the factor by which $\frac{V_s}{[E]}$ is changed by changing [S]. The derivation of the equation has been presented in Section 4.3.7.

A standard curve (as illustrated in Figure 4.10b) to obtain 'k' was prepared for a particular batch or enzyme preparation with known NaFa standard. A sample containing an unknown concentration of NaFa could be incubated (under the same conditions as used for obtaining the standard curve) with an enzyme preparation of known concentration $[E_x]$ and the initial rate of defluorination, (V_x), determined. By substituting $V_x[E_x]^{-1}$ into the above equation, the concentration of NaFa in the sample could be determined, or the concentration could be read out from the standard curve.

Considerations - Both methods could be potentially very useful for bioassaying residual NaFa in NaFa-treated baits. The enzymatic method has an advantage over the cellular method in terms of rapidity since in the former method only the initial rate of F^- released needs to be measured whereas the latter method requires total defluorination. Both these biological methods suffer from the disadvantage of being sensitive to inhibitors such as heavy metals and thiol-alkylators which would have to be removed prior to bioassay. Furthermore both cells and enzymes deteriorate during storage and this would require that for any one batch of enzyme preparation, a standard curve has to be prepared for each new set of bioassays. The rate of deterioration may be reduced by storing the preparations at $-20^{\circ}C$.

Both methods are based on F^- estimation, and the rapidity is made possible by the use of an F^--specific electrode. Consequently the range of NaFa concentrations that could be determined depends on the sensitivity of the electrode which normally has a lower limit at 10^{-6} M F^-. The rapidity and the efficiency of the method depends also on the extraction of bound F^- from test materials.

It is assumed that for every mole of F^- released and detected, one mole of NaFa is present in the incubation mixture of cells or enzymes with the sample.

CHAPTER FIVE

PHYTOTOXICITY STUDIES WITH *Chlorella* SP. AND DUCKWEEDS

5.1 INTRODUCTION

As outlined earlier (Chapter 1.6) NaFa is highly toxic to warm blooded animals and insects. Available evidence suggests that plants are, comparatively speaking, less sensitive to NaFa. For instance, there are plants which naturally synthesise and accumulate fluoroacetate in concentrations high enough to kill animals grazing on them. Serious stock losses due to this type of poisoning led to the discovery of fluoroacetate; first in *Dichapetalum cymosum* (Marais, 1944), and later in *D. toxicarium* (Peters *et. al.*,1960); *Palicourea marcgravii* (de Oliveira, 1963), *Gastrolobium grandiflorum* (McEwan, 1964), *Acacia georginae* (Murray *et. al.*, 1961; Oelrichs & McEwan, 1961,(1962) and other species of *Gastrolobium* and *Oxylobium* (Aplin, 1971). Studies on the use of NaFa as a systemic insecticide revealed that broad bean plants showed injury only at NaFa concentrations 50-100 times higher than that which killed aphids (David, 1950). However there are some plants which are susceptible to NaFa poisoning. The damage to plants ranges from leaf necrosis, slight to extensive wilting and eventual death at higher concentrations. Variation in susceptibility, as seen among animals, is also observed among different plants: leaf necrosis developed in turnip plants grown in the presence of 1.0 mM NaFa in nutrient medium (David & Gardiner, 1954), and similar damage occurred to soybean plants, *Glycine max* (Cheng *et. al.*,1968) at 5.0 mM, and in *Helianthus annuus*, *Lolium perenne* and *Achillea millifolium* at 10 mM NaFa (Cooke, 1976c). Plants are thus variable in their response to the presence of NaFa.

In the present work, the effect of NaFa and NaF (one of the breakdown products of NaFa) on the growth of some plants was studied: *Chlorella* sp. isolated from soils; *Lemna minor*, and *Spirodela oligorrhiza* which are common in waterways; and also of interest, the European species of giant duckweed *S. polyrrhiza*. These plants were chosen for study because of their likelihood of coming in contact with NaFa when applied as pesticide, and their ease and convenience of handling under aseptic conditions.

5.2 METHODS AND MATERIALS

5.2.1 Plants: Isolation, Purification and Maintenance

a. *Chlorella* sp. - *Chlorella* sp. was isolated from enrichment cultures inoculated with soils (refer Chapter 2), using standard microbiological techniques as recommended by Lewin (1959). The procedure involved plating out a small sample drawn from an enrichment culture on NaFa mineral salts agar medium, and incubating in the light at 25°C for 4 days. A small algal colony was selected and suspended in sterile distilled water. Further serial dilution of the suspension was made by transfer of a small drop into a small drop of water placed on an agar plate using a sterile pasteur pipette. Successive withdrawals and transfers onto further drops of water were repeated until only a few cells were left in the final drop of water. Dilution was ascertained by examination under ligh microscope. The final drop was streaked on a fresh agar plate and incubated for 5 days. An inoculum from the smallest algal colony appeared on this plate was further streaked on to a fresh agar plate. This was repeated until a pure algal isolate, free from bacterial contamination, was obtained (as ascertained by light microscopic examination). *Chlorella* sp. was maintained on either NaFa-medium or basal medium.

b. *Lemna minor* - *Lemna minor* was isolated from a drainage ditch near Lewis Pass (South Island). The duckweeds were surface-sterilised in ¼ strength commercial sodium hypochlorite (domestic bleach)solution for periods ranging from 30 seconds to 5 minutes and subsequently rinsed in 5 changes of sterile distilled water prior to transfer to basal medium for incubation in the light at 20°C. The duckweeds which survived hypochlorite sterilisation for the longest period (3 min) were subcultured in basal medium and checked for bacterial contaminants by microscopic examination of the culture medium. Bacteria-free cultures were used for study.

c. *Spirodela oligorrhiza* and *S. polyrrhiza*

An axenic culture of *S. oligorrhiza* was obtained from Dr. E.G. Bollard (Department of Scientific and Industrial Research, Auckland) and *S.polyrrhiza* was obtained from Dr J. McWha

(Department of Botany, University of Canterbury). The cultures were maintained in basal medium, in the light at 20°C.

Cultures of *Chlorella* sp. and duckweeds were subcultured every 2 weeks. Growth medium was sterilised by autoclaving at 120°C for 15-20 minutes.

5.2.2. Determination of Growth and Conditions of Growth

The growth of *Chlorella* sp. was determined by cell counts using a haemacytometer. Growth of duckweeds was followed by counting the number of fronds which were 2/3 or more of the maximum size of a mature frond. Cultures were incubated in constant light (200 μ Einsteins) at 20°C.

5.2.3a General Procedure for studies on growth of duckweeds - 30 ml aliquots of basal medium dispensed into 100-ml conical flasks were each inoculated with 6 mature fronds of *L. minor*, *S. oligorrhiza* or *S. polyrrhiza*. For each treatement (NaF or NaFa, NaF+NaFa), there were 5 replicates.

The effect of NaFa was examined from 0.0 - 1.0 mM; the NaF range was 0.0 - 20 mM. Possible interaction of NaF and NaFa was also examined from combination of various concentrations of NaF (0-10 mM) and NaFa (0.0-0.1 mM).

b. Procedure for Studies of growth of *Chlorella* sp.- 10-ml aliquots of basal medium containing either 20 mM NaFa or sodium acetate were inoculated with 0.1 ml cell suspension of *Chlorella* sp., and the final cell density of each growth tube was 4×10^3 cells ml^{-1}. Four experimental conditions were studied with 6 replicates each:

(a) Light, aerobic;
(b) Light, anaerobic (air supply was reduced by layering sterile paraffin oil over the inoculated medium);
(c) Dark, aerobic; and
(d) Dark, anaerobic.

All growth tubes were incubated at 25°C for 2 weeks.

5.2.4 Respirometric Studies with Cells of *Chlorella* sp.

Cells of *Chlorella* sp. grown in basal medium were harvested (by centrifuging at 5,000 g for 10 minutes) from 7-day old

cultures, washed several times with, and suspended in phosphate buffer pH 7.2. The density of the cell suspension was adjusted to 8.0×10^5 cells ml^{-1}. Oxygen uptake was measured by direct Warburg method (Umbreit *et. al.*, 1964) using a Gilson differential respirometer. To each 15-ml Warburg flask were added: 1.5 ml cell suspension into the central compartment, 1.5 ml 20 mM NaFa or distilled water into the sidearm, and 0.2 ml 15% KOH into the centre well together with a pleated strip of filter paper to facilitate CO_2-trapping. The temperature of assay was 25°C and the system was equilibrated for 40 minutes prior to tipping contents of the sidearm into the central compartment.

5.3 RESULTS

5.3.1 Effect of NaFa on *Chlorella* sp.

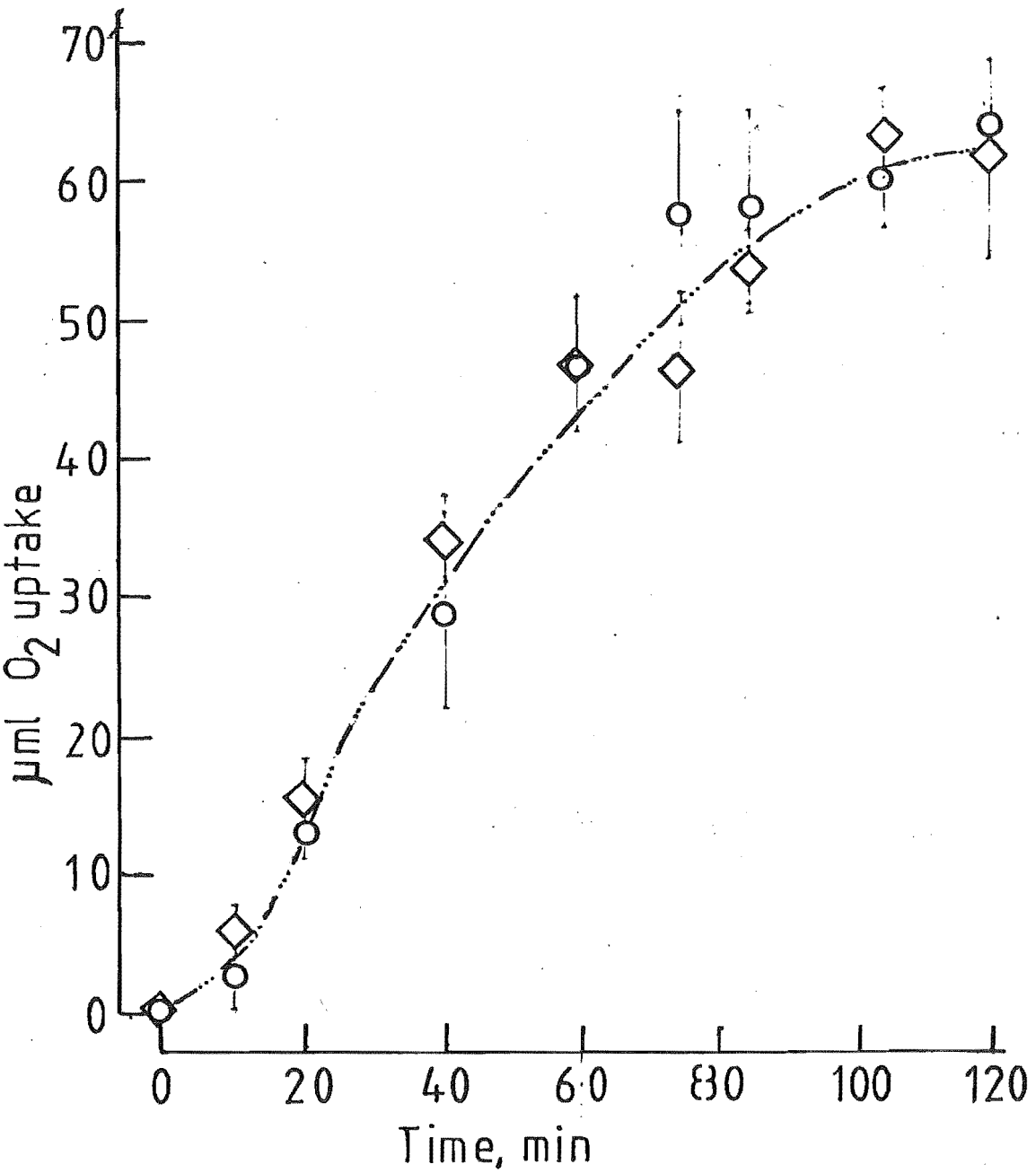
Good growth of *Chlorella* sp. was observed in 20 mM NaFa or acetate-supplemented basal medium incubated in the presence of light and air. The total cell yield in NaFa-supplemented medium (7×10^5 cells ml^{-1}) was slightly lower than in the acetate-supplemented medium (8.7×10^5 cells ml^{-1}). Concentration of F^- in NaFa-supplemented culture medium was less than 10 µM as measured by means of an F^- - specific electrode previously calibrated to read F^- concentration between 10 µM and 100 µM. Growth in the dark with or without air was not evident in either NaFa - or acetate-supplemented medium. After prolonged incubation in the dark, the cells became translucent, losing all their pigments. These results suggest that *Chlorella* sp. is an obligate photoautotroph requiring both light and CO_2 for growth.

Further studies on the rate of O_2-uptake of *Chlorella* sp. cells in the presence of NaFa was similar to that of endogenous respiration (in the absence of NaFa) as shown in Figure 5.1, thus suggesting that respiration of the cells of *Chlorella* sp was not inhibited by NaFa at the concentration used (20 mM).

5.3.2 Effect of NaFa on Growth of Duckweeds

a. Growth of the Duckweeds - *Spirodella oligorrhiza*, *S. polyrrhiza* and *Lemna minor* was followed by counting the number of fronds each day. The growth of each species of duckweeds followed the normal

FIG 5.1 EFFECT OF NaFa ON OXYGEN UPTAKE OF CELLS OF SP. *Chlorella*.



The incubation mixture contained 1.5 ml cell suspension of *Chlorella* sp. and 1.5 ml NaFa (20 mM), incubated at 25°C. The amount of O_2 uptake measured. Results are averages of 4 replicates. Control was set up without NaFa.

Incubated with NaFa ○—···—○

Control (without NaFa) ◇—···—◇

growth pattern - exponential increase in frond number as shown in Figures 5.2a-5.2c. The growth pattern is expressed by (Sokal & Rohlf, 1969): Equation (VIa):

$$N_t = N_o \cdot e^{bt} \text{ ; or } \ln N_t = \ln N_o + b \cdot \ln t \text{ ; or}$$

$$\log N_t = \log N_o = b't \quad \text{where}$$

N_t = number of fronds present at time t (days)

N_o = initial number of fronds introduced; and

b' = b (log e) or growth rate constant which is the slope of the growth curve shown in Figures 5.2a-5.2c.

Thus the assessment of the effect of NaFa and/or NaF was based on determinations of growth rate constants. The range of NaFa concentration examined was 0-1.0 mM.

The inhibitory effect of NaFa on the duckweeds was shown by increasing reduction in growth constants as the concentrations of NaFa in the medium was increased. To facilitate the comparison of the effect on different species of duckweeds, the growth rate constants were plotted against the concentration of NaFa as shown in Figure 5.3, expressed by the equation:

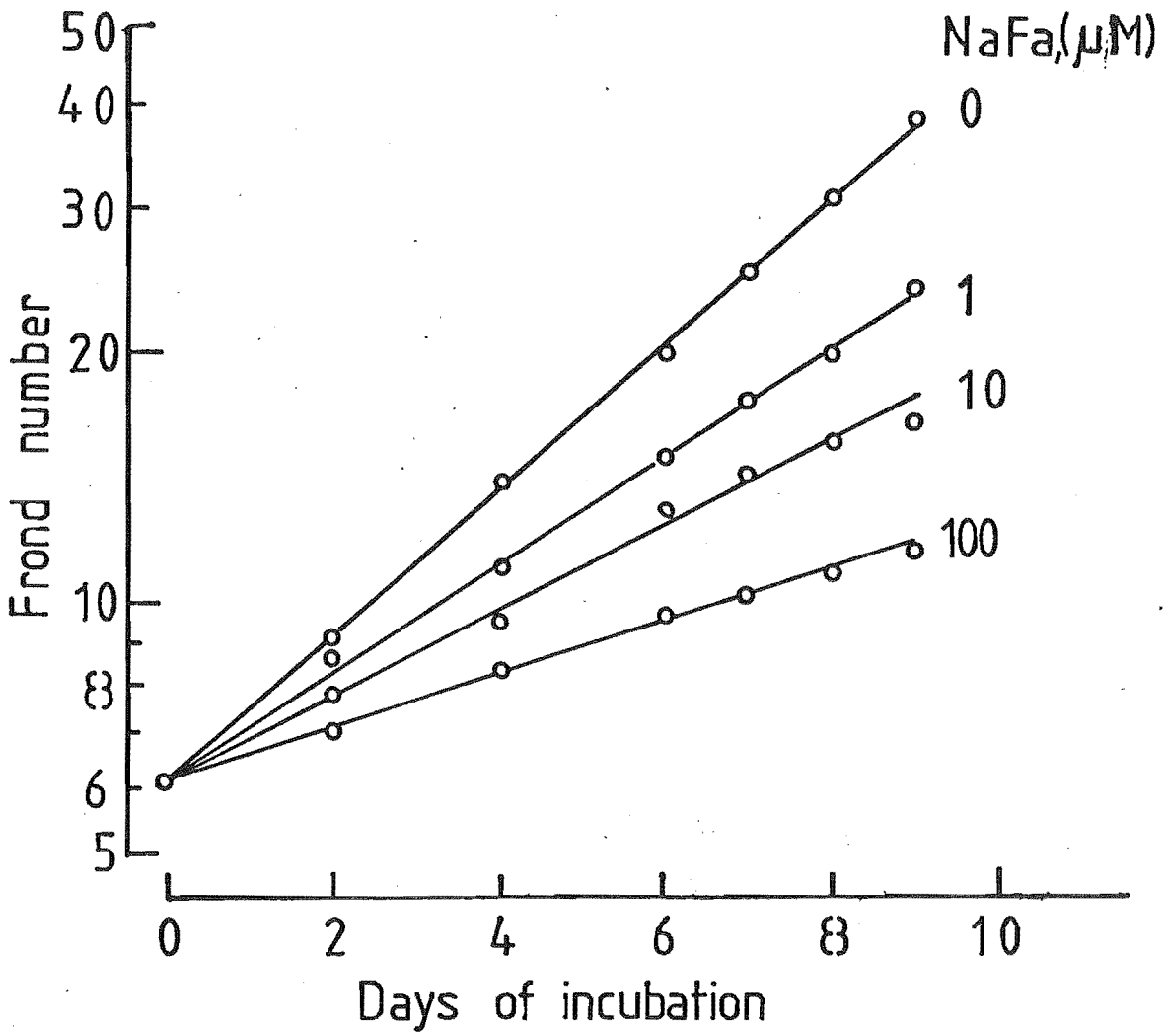
Equation (VII) $b_{fa} = a_{fa} + q \cdot \log [\text{NaFa}]$ where

b_{fa} = growth rate constant in the presence of NaFa at concentration [NaFa];

a_{fa} = growth rate constant when log [NaFa] = 0;

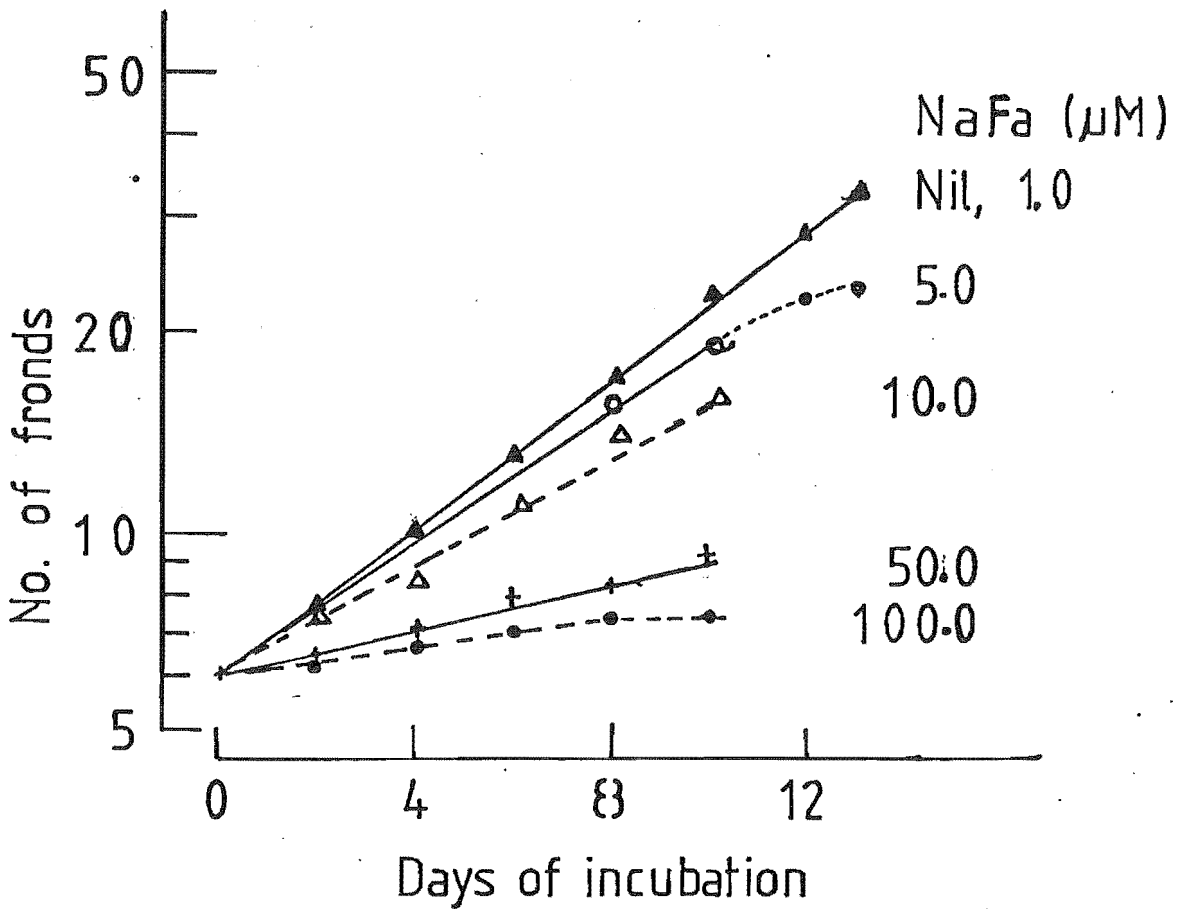
and q is the slope of the line relating growth rate constants (b_{fa}) to NaFa concentration, it thus gives a quantitative measure of the sensitivity of the duckweed to increasing concentration of NaFa in the growth medium. The q values for each species of duckweed studied are presented in Table 5.1. On the basis of q values calculated, *S. polyrrhiza* is 1.6 and 1.2 times more sensitive to fluoroacetate (within the concentration ranges 50-100 µM NaFa) than *S. oligorrhiza* and *L. minor* respectively (significant at 0.05 probability level). At the lower concentration range of NaFa $\leq$ 5.0 µM, *S. polyrrhiza* was comparatively less affected than *S. oligorrhiza* and *L. minor* but complete suppression of growth occurred at 500 µM NaFa for both *S. oligorrhiza* and *L. minor*.

FIG 5.2A EFFECT OF NAFA ON GROWTH OF *S. oligorrhiza*



Cultures of the duckweeds were incubated in the light at 25°C. Results expressed are averages of 5 replicates. Results for NaFa at 5, 50 and 500 μM were not shown.

FIG 5.2B EFFECT OF NAFA ON THE GROWTH OF *S. polyrrhiza*



Culture conditions as described in Fig 5.2a.

FIG 5.2.C EFFECT ON *L. minor*

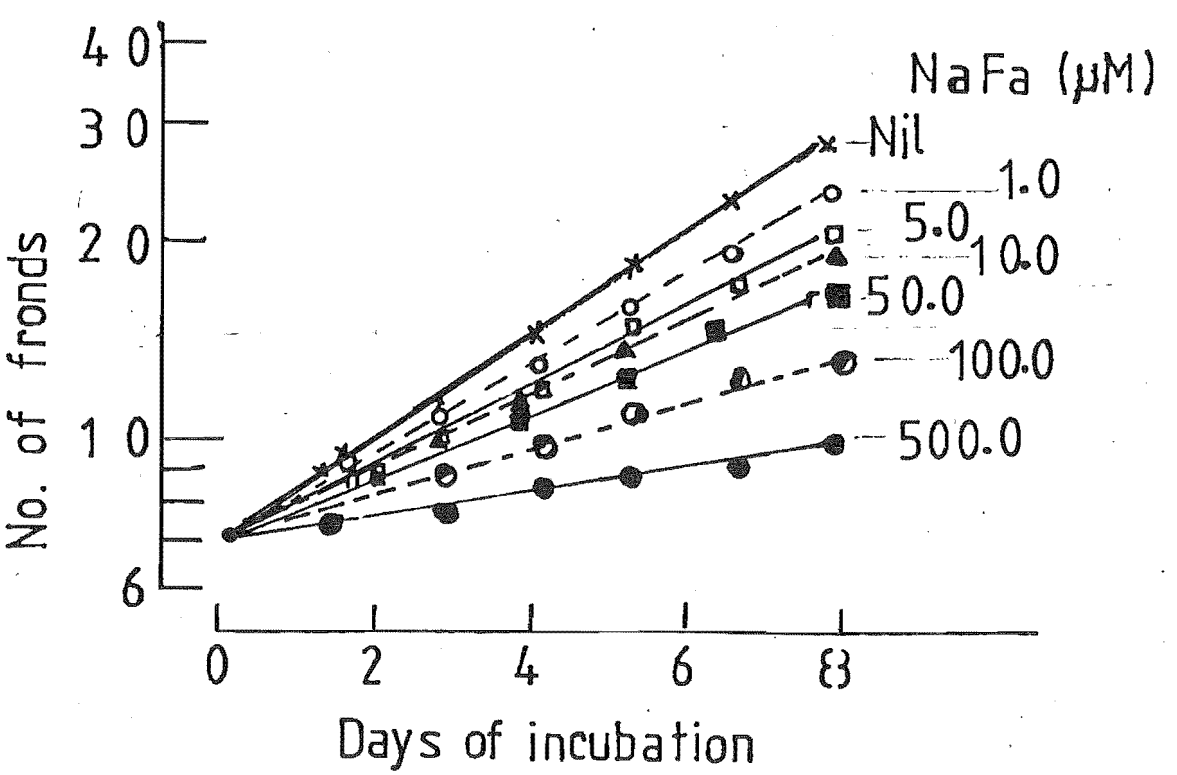
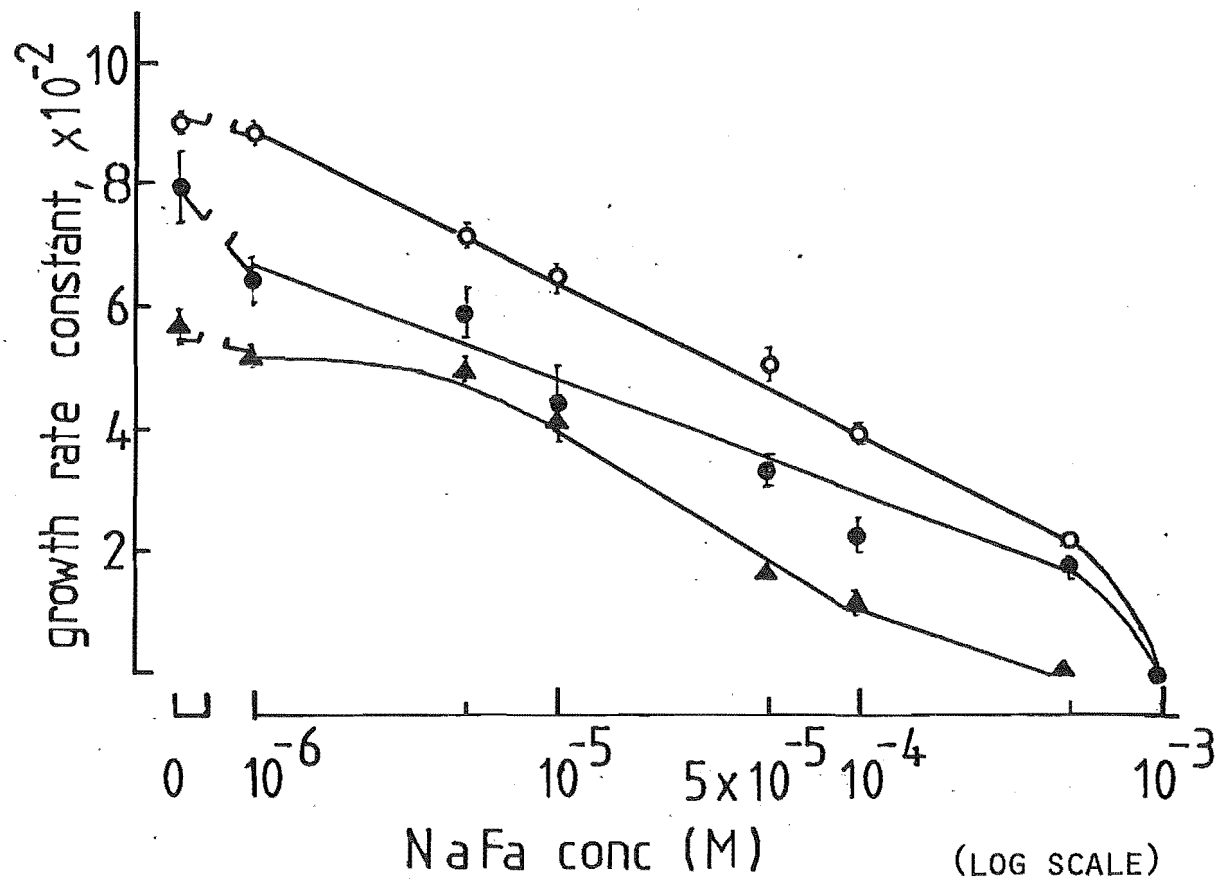


FIG 5.3: SENSITIVITY (q) OF DUCKWEEDS TO NAFA



Duckweeds	q, sensitivity ± std deviation	NaFa (μM) range, [S]
• S. oligorrhiza	0.018 ± 0.001	$1 \ll [S] \ll 500$
▲ S. polyrrhiza	0.029 0.002	$5 \ll [S] \ll 100$
○ L. minor	0.024 ± 0 001	$1 \ll [S] \ll 500$

Table 5.1: Sensitivity of Duckweeds (Dosage-Response Relations)

Species of duckweeds	a_{fa}	q ± S.D.	NaFa Concentration range (μM)
S. oligorrhiza	0.070	0.018±0.001	1μM ≤ NaFa ≤ 500 μM
S. polyrrhiza	0.058	0.029±0.002	5μM ≤ NaFa ≤ 100 μM
L. minor	0.089	0.024±0.001	1μM ≤ NaFa ≤ 500 μM

S.D. = standard deviation

b. Effect of inoculum density on Sensitivity of *S.oligorrhiza* to NaFa

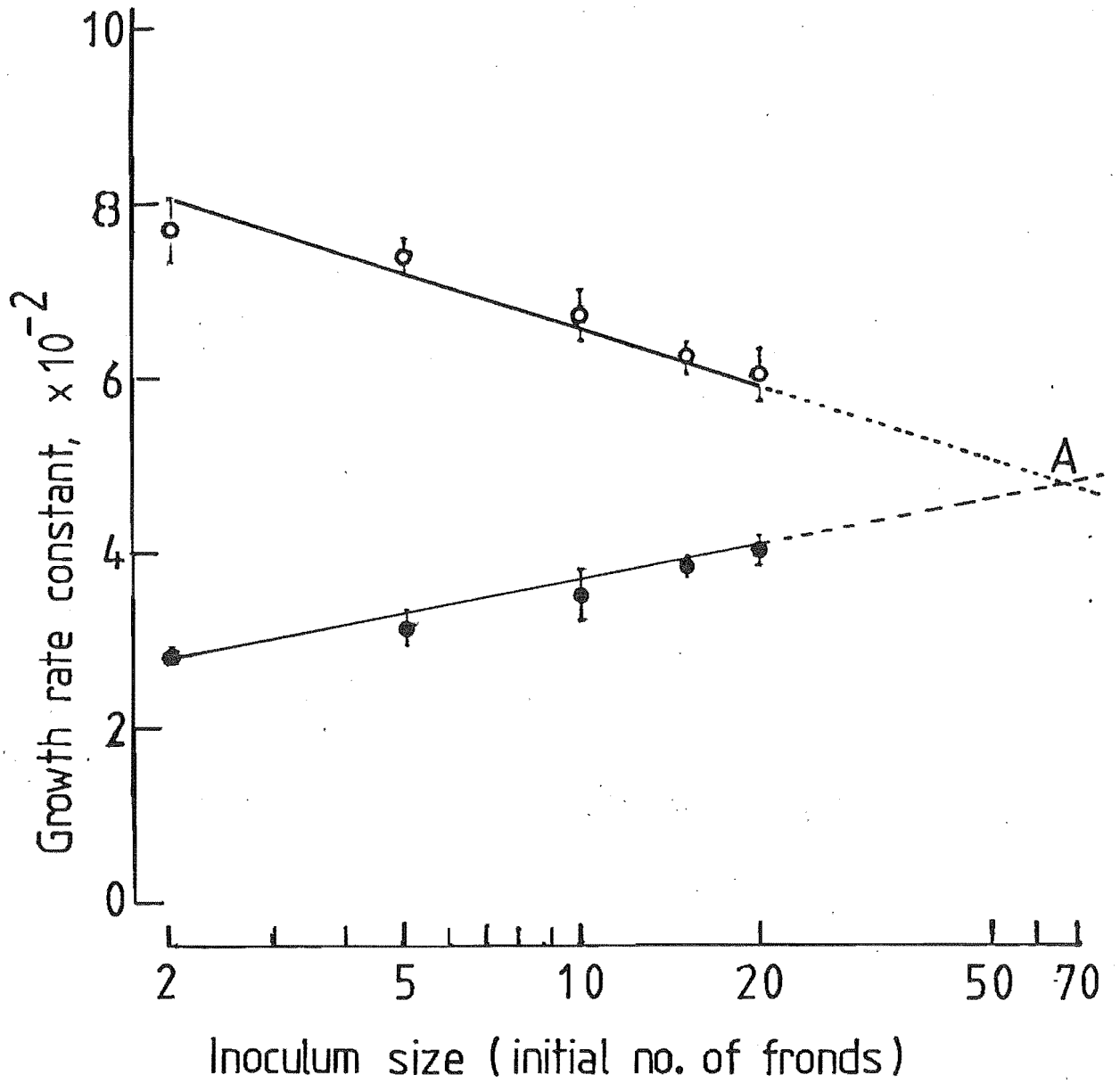
The relative decrease in sensitivity of *S. polyrrhiza* at NaFa concentrations ≤ 5.0 μM and increase in sensitivity at higher concentration compared to the other two species of duckweeds may be the result of the differences in the frond size (cf. frond size of *S. oligorrhiza* and *L. minor*), and hence uptake of NaFa. A frond with a larger surface area could absorb more NaFa from the medium than could a smaller frond. Consequently the medium could be diluted to a greater extent by the former and rendered less toxic. This dilution effect was tested by using an increasing inoculum density (2-20 fronds, introduced initially) in the presence of 50 μM NaFa and in its absence. Growth rate constants in the presence of 50 μM NaFa increased with increasing inoculum density as shown in Figure 5.4, whereas growth rate constants decreased with increasing inoculum density in the control. The relationship could be described by the general equation,
$y = a + \log(x)$, as shown in Fig. 5.4

Equation (VIIIa) $b_{fa} = 0.0191 + 0.0163 \pm 0.0008 \log (N)$ in the presence of NaFa,

Equation (VIIIb) $b_o = 0.0896 - 0.0226\ 0.002 \log (N)$ in the absence of NaFa where b_{fa} (in the presence of NaFa) or b_o (in the absence of NaFa) is the growth rate constant at inoculum density, (N).

Thus in 50 μM NaFa medium, the growth rate constant was increased by 0.0163 unit for every 10-fold increase in inoculum density whereas in the absence of NaFa, the growth rate constant was decreased by 0.0226 units for a similar increase in inoculum density. Thus these results show that the toxicity of NaFa to

FIG 5.4: EFFECT OF INOCULUM SIZE ON THE SENSITIVITY OF *S. oligorrhiza* TO NAFA



Culture conditions were as described in Fig 5.2a. The apparent decrease in susceptibility of *S. oligorrhiza* to 50 µM NaFa as a result of increasing the number of starting fronds is shown. Results are averages of 5 replicares.

Treatment		slope
Control	o——o	- 22.6 ± 2.1
With 50 µM NaFa	•——•	16.3 ± 0.1

A : hypothetical inoculum size, ≃ 65 fronds.

S. oligorrhiza is lessened if the duckweed is present in large numbers, and that the effect due to overcrowding was counter-balanced by the lower growth rate constants in the presence of NaFa.

Because the response of duckweeds to increasing inoculum density in NaFa medium was directionally opposite that in its absence, a frond density could be eventually reached when the decrease in growth rate constant due to overcrowding would equal the apparent increase in tolerance (increasing growth rate constant) of the duckweed to NaFa. At this density, b_{fa} would be equal to b_o of the two equations above, the density could be calculated by simultaneous solving of the two equations and was found to be 65. The inhibition in growth due to NaFa can theoretically be obscured by the overcrowding effect at (N) = 65. Hence, while the sensitivity of duckweeds to NaFa suggests their potential as an indicator organism for toxicity of waterways contaminated with NaFa, the population dependent sensitivity may counteract the effect.

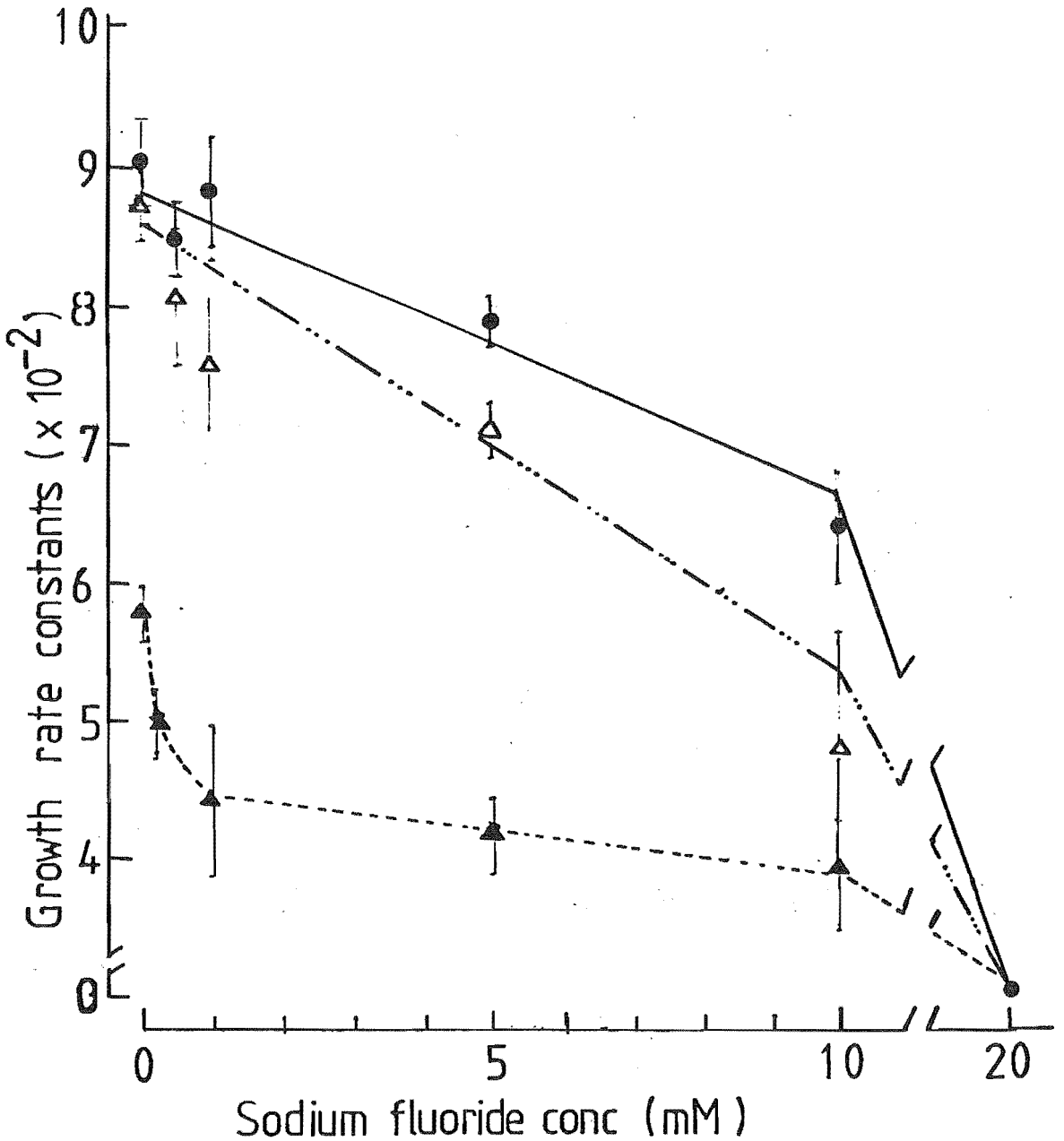
5.3.3 Effect of Sodium Fluoride on the Growth of Duckweeds

The effect of NaF was also studied because it is a breakdown product of NaFa and consideration of NaFa as a pollutant should therefore be accompanied by consideration of NaF. The growth of duckweeds in the presence of NaF concentration ranging from 0.0 to 20 mM was studied. The growth of all 3 species of duckweeds was retarded in the presence of NaF and completely inhibited at 20 mM NaF. As shown in Figure 5.5, the sensitivity of the 3 species of duckweeds of NaF differed in magnitude within the range of NaF concentrations to which the duckweeds were exposed. At NaF $\leq$ 1.0 mM growth rate constants of *S. oligorrhiza* and *L. minor* were similar to those in the absence of NaF, whereas growth of *S. polyrrhiza* was considerably retarded. At NaF $\geq$ 1.0 mM, *S. polyrrhiza* became relatively less sensitive while the growth of *S. oligorrhiza* and *L. minor* was retarded. At 20 mM NaF, growth of all duckweeds was completely suppressed and the fronds eventually died.

These results indicate that NaFa was considerably more toxic than F^- to all the duckweeds studied.

Physiological effect of NaFa and NaF on duckweeds - The progress

Fig 5.5 Effect of sodium fluoride on duckweeds



Culture conditions were as described in Fig. 5.2a. Results expressed are averages of 5 replicates

DUCKWEEDS

L. minor

S. oligorrhiza

S. polyrrhiza

of physiological damage of the duckweeds caused by NaFa and NaF were clearly different although both toxic agents caused premature ageing of fronds as evidenced by earlier senescence compared to control cultures. The earliest symptons of NaFa poisoning of the duckweeds was chlorosis which spread inwards from the margin of the fronds towards the budding centre. Chlorosis was followed by browning which proceeded in the same direction and the fronds eventually bleached and died. NaF caused early bleaching at acute concentrations $\geq$ 10 mM; and at lower concentration, chlorosis preceded bleaching. Browning was not observed in fronds treated with NaF.

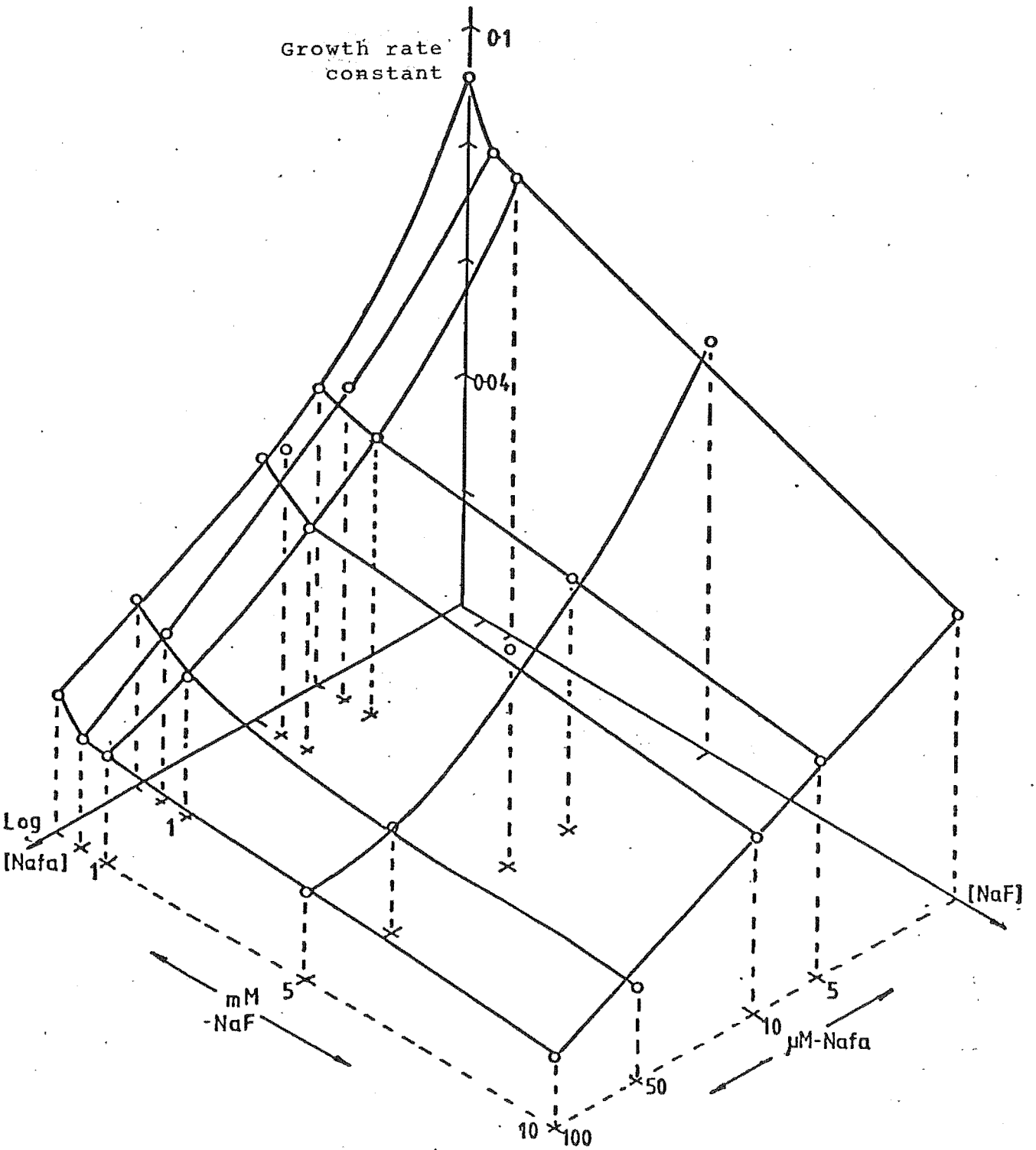
Observations were made also on the size of fronds which survived and grew in the presence of either NaFa or NaF. In an acutely toxic situation with NaFa $\geq$ 0.1 mM, the fronds were comparatively smaller; about one third of the size of a healthy control frond but at lower NaFa concentrations, differences in the size of fronds was not evident. By contrast limitations in the development of frond size was more distinct in the presence of NaF at all concentrations examined.

5.3.4 Possible Interactive Effects of NaFa and NaF

The possibility of an increased inhibitory effect was investigated using *S. oligorrhiza* grown in the presence of different concentrations of both NaFa (0, 5, 10, 50, 100 µM) and NaF (0.1, 5, 10 mM) with 5 replicates for each combination of NaFa and NaF. The results are shown in Figure 5.6 and summarised in Table 5.2. Data was computer analysed using a "Multivariate analysis of variance" of the growth rate constants from the twenty-five combinations of NaFa/NaF treatments and Duncan's Range Test (Appendix IIa and IIb).

The results of this multivariate analysis of variance of the growth rate constants showed that reductions in growth rate due to NaFa or NaF were significant; and that an interactive or multiplicative effect was not evident, suggesting that NaFa and NaF exerted their inhibitory effects independently. Further analysis of results by Duncan's Range Test showed that

FIG 5.6 POSSIBLE INTERACTIVE EFFECT OF NaFa AND NaF ON THE GROWTH OF *S. oligorrhiza*.



Culture conditions as described in Fig 5.2a. The effect of different concentrations of NaF/NaFa on the growth rate constant of *S. oligorrhiza* is shown.
Results expressed are averages of 5 replicates.

Table 5.2: Effect of Co-presence of NaF and NaFa on Growth Constant of *S. oligorrhiza*

Concentration of NaFa (M)	Growth rate constants±standard deviation				
	Concentration of NaF (M)				
	0.0	$5x10^{-4}$	$1.0x10^{-3}$	$5.0x10^{-3}$	$1.0x10^{2}$
0.0	0.086±0.005	0.081±0.001	0.078±0.003	0.071±0.002	0.048±0.002
$5.0x10^{-6}$	0.058±0.004	0.054±0.002	0.047±0.008	0.044±0.001	0.037±0.002
$1.0x10^{-5}$	0.045±0.006	0.049±0.001	0.038±0.003	0.037±0.002	0.031±0.002
$5.0x10^{-5}$	0.033±0.002	0.027±0.001	0.023±0.003	0.020±0.001	0.018±0.003
$1.0x10^{-4}$	0.023±0.003	0.018±1.003	0.015±0.002	0.016±0.003	0.011±0.001

Results are averages of 5 replicates

(1) The differences in growth rate constants of *S. oligorrhiza* due to NaF alone at concentrations $\leq$ 1.0 mM were insignificant, but significant at higher concentrations (5% probability level);

(2) At 100 µM NaFa, the reduction in growth rate constants due to increase in NaF concentration was insignificant at 5% probability level. At NaFa $\leq$ 100 µM, the reduction in growth rate constant due to every 10-fold increase in NaF was significant; and

(3) At all concentrations of NaF, the reduction in growth rate constant due to every 10-fold increase in NaFa was significant.

In summary, these results indicate that the presence of both NaFa and NaF is slightly more inhibitory (significant at 7% probability level) than in the presence of either toxic agent singly; and the increase in toxicity due to increase in NaFa at all concentrations of NaF was greater than the increase in toxicity due to equal interval of increase in NaF at all concentrations of NaFa.

5.4 DISCUSSION

5.4.1 Effect on NaFa on Plants

Plants vary in their response to treatment with NaFa and all the duckweeds studied were highly sensitive to NaFa. The severity of NaFa toxicity to duckweeds ranged from invisible damage to fronds with reduced growth rate constant at NaFa $\leq$ 0.5 mM ($\leq$ 12 µg NaFa per frond) to extensive chlorosis of the fronds with complete cessation of growth at NaFa $\geq$ 0.5 mM. Progressive marginal browning occurred in fronds treated with NaFa but not NaF. Similar qualitative differences were also observed by Cooke (1976c) on the leaves of *Helianthus annus* grown in NaFa-, and in NaF-supplemented medium. The three species of duckweeds varied in their sensitivity to Nafa; *S. polyrrhiza* was 1.6 and 1.2 times more susceptible to NaFa poisoning than *S. oligorrhiza* and *L. minor*. Inhibition of growth of these duckweeds was concentration-dependent, implying that the action of NaFa may lie in the inhibition of a rate-limiting step in an important metabolic pathway. The extensive chlorosis observed may be the result of a general reduction of metabolic activity.

Sir Rudolph Peters (1957) expounded his theory of "lethal synthesis" to explain the mechanism of NaFa poisoning of animals.

This theory proposed the conversion of fluoroacetate to fluoroacetyl CoA which on subsequent condensation with oxaloacetate formed fluorocitrate. Fluorocitrate competes with citrate for the active site on the enzyme, aconitase, thus blocking TCA cycle (Tricarboxylic acid cycle) at that point.

The TCA cycle is the major respiratory pathway for the generation of ATP and intermediates for the biosythesis of amino acids, fatty acids, carbohydrates and proteins. Although attempts have not been made to isolate fluorocitrate from the duckweeds grown in the presence of NaFa, it has been found in some plants - *Medicago sativa* and *Agropyron cristatum* (Lovelace *et. al.*, 1968); *Glycine max* (Cheng *et. al.*, 1968) and more recently in the blue-green algae, *Gloeocapsa* (Gallon *et al.*, 1978). Furthermore, there is evidence that some plant aconitases are sensitive to fluorocitrate: the aconitase isolated from *Glycine max* (Cheng *et. al.*, 1968) was only half as sensitive as that isolated from pig's liver, but the aconitase isolated from *Dichapetalum cymosum*, which synthesises and accumulates fluoroacetate, was sensitive to inhibition by fluorocitrate (Eloff & Von Sydow, 1971). Thus the reduced growth rate and/or lethal effect of NaFa on the duckweeds could be due to the lethal synthesis of fluorocitrate and the differential sensitivity of the 3 species of duckweeds could result from varying sensitivity of their aconitase to fluorocitrate.

By contrast, the soil alga studied, *Chlorella* sp. was found to be remarkably tolerant to NaFa. *Chlorella* sp. was able to grow and multiply normally in the presence of 20 mM NaFa provided light and air were available but was incapable of heterotrophic growth on either NaFa or sodium acetate. It appears that, under the conditions studied, the cells of *Chlorella* sp. depended on photosynthesis for growth in the presence or absence of NaFa. Tolerance of plants to NaFa is not uncommon, as seen with plants which synthesise NaFa (Refer 5.1). Several explanations have been offered to explain the tolerance of plants to NaFa and may be categorised thus:

(a) Specificity of the enzymes: aconitase, thiokinase and/or citrate synthetase which are involved in TCA cycle;
(b) Prior detoxification of NaFa by defluorination catalysed by a plant fluorohydrolase;
(c) Incorporation of fluoroacetate, or fluorocitrate, if formed into lipids.

Peters (1972) suggested that plant aconitases were comparatively more specific to citrate, and thus less sensitive to fluorocitrate. Evidence in support of this proposal was the discovery of a multiplicity of aconitase isozymes of different sensitivity in a plant tissue preparation (Peters, 1972) and the report that aconitase extracted from *Acer platanus* and pea seedlings were 2000 times less sensitive to fluorocitrate than those from animal tissues (Treble *et. al.*, 1962). However this cannot account for the tolerance of *D. cymosum* to fluoroacetate which has been estimated to be present at a concentration as high as 789 mg kg^{-1} (dry wt) tissue (Marais, 1944) and the observation (Eloff & Von Sydow *et. al.*, 1971) that aconitase from leaves of *D. cymosum* was sensitive to inhibition by fluorocitrate. Based on respirometric studies, Eloff & Von Sydow (1971) found that the O_2 uptake of leaf discs of *D. cymosum* was not inhibited in the presence of fluoroacetate but was inhibited in the presence of fluorocitrate. This led Eloff & Von Sydow to suggest that the tolerance of plants to NaFa may be attributed to the specificity of acetate thiokinase so that fluoroacetyl CoA was not formed and/or that citrate synthetase was highly specific so that condensation of fluoroacetyl-CoA (if formed) with oxaloacetate did not take place. This seems plausible if such a specificity of acetate thiokinase and/or citrate synthetase is demonstrated. Respirometric studies with *Chlorella* sp. in the presence of NaFa showed no inhibition of O_2 -uptake, suggesting the cells may be incapable of synthesising fluorocitrate.

Another possibility considered was the actual defluorination of fluoroacetate by plants as suggested by Preuss *et. al.*,(1968) and Preuss & Weinstein (1969). Preuss & Weinstein (1969) reported that peanut seedlings incubated in the presence of fluoroacetate converted 29% of the organic F into F^-, while seedlings of *Acacia georginae* (which naturally synthesises fluoroacetate) metabolised only 33% of fluoroacetate supplied (Preuss *et. al.*, 1968). Defluorination of fluoroacetate was also reported to occur in

H. annuus (Cooke, 1976c), lettuce (Ward, 1973) and in *A. georginae* (Hall, 1974). The possibility that the *Chlorella* sp. studied may be able to defluorinate NaFa was also examined by measuring F^- concentration in the culture medium. Growth of *Chlorella* sp. was evident but no release of F^- into the medium was detected. With the exception of Preuss *et. al.*, (1968) and Preuss & Weinstein (1969)much of the early work on plant metabolishm of fluoroacetate by plants was not carried out under aseptic conditions. Consequently, any claims of defluorination on the basis of positive finding of inorganic F^- in the plants is open to question. Fluoroacetate can be easily broken down by micro-organisms as demonstrated in the past (Goldman, 1965; Kelly, 1965; Tonomura *et. al.*, 1965) and confirmed in the present work. The inorganic F^- found in the plants could have been derived from soils or culture medium in which fluoroacetate was previously defluorinated and F^- subsequently taken up by the plants. This is particularly feasible in view of Hall's finding that 84.5% of the F^- detected was in the root portion of *A. georginae* seedlings transplanted in to NaFa-treated soils(Hall, (1974). Thus the eventual proof of the ability of plants to cleave the C-F bond of fluoroacetate lies in the isolation of the defluorinating enzyme (fluorohydrolase) from these plants. As yet there are no reports of such an attempt.

Plants which are able to defluorinate fluorocitrate have not been reported although evidence has been found that a soil pseudomonad could defluorinate fluorocitrate (Kirk & Goldman, 1970). It has also been found in present work that DL-fluorocitrate spontaneously defluorinates. It may be possible that defluorination of fluoroacetate in plants as claimed by the above workers, may be the result of defluorination of fluorocitrate rather than fluoroacetate. However fluorocitrate is not the only product arising from fluoroacetate since higher fluoro-organic compounds such as ω-fluorooleic and fluoropalmitic acids have been found in the seeds of *Dichapetalum toxicarium* (Peters *et. al.*, 1960), and found to be incorporated into lipids in plants incubated with NaFa (Preuss *et. al.*, 1968a; Gallon *et. al.*, 1978). Biochemical analysis of cells of *Chlorella* sp. incubated with NaFa was not attempted in the present study so that the possibility that *Chlorella* sp. escapes NaFa intoxication by converting it into inert compounds as above is not known, but it is clear that it does not defluorinate NaFa.

In view of the finding that *Chlorella* sp. was able to grow in the presence of NaFa provided air and light were available and reports that plants are generally tolerant to NaFa, I would like to offer another explanation for the relative insensitivity of plants to NaFa. This is based on the relative importance of the TCA cycle and the plant's photosynthetic systems for the provision of ATP, reductants and carbon skeletons for tbe biosynthesis of carbohydrates and proteins. It is suggested that the tolerance of *Chlorella* sp., to NaFa may be conferred by its ability to photosynthesise and thus derive its energy and raw material for growth from photosynthetic phosphorylation and photosynthates. The role of photosynthesis in conferring tolerance is hereby elaborated further.

There are three major pathways of energy (as ATP) generation: glycolysis, the oxidative pentose phosphate pathway and the TCA cycle. Plants possess the additional pathway for photosynthetic phosphorylation and the role of these routes for ATP and reductant (NADPH) generation during the growth of unicellular algae has been reviewed by Raven (1976). The evidence he gathered showed that in phototrophically growing cells the capacity for glycolysis, oxidative pentose phosphate pathway and TCA cycle was low, as determined from examination of the activity of certain key enzymes such as phosphoglycerate kinase, pyruvate kinase (glycolysis), glucose-6-phosphate and 6-phosphogluconate dehydrogenases (Pentose phosphate pathway). Further evidence for the relative unimportance of respiration in phototrophically grown cells came from quantitative microscopy: mitochondria (TCA cycle) comprised only 5% of the cell volume of phototrophically grown cells as compared to 20% found in heterotrophically grown cells of *Chlamydomonas* or *Chlorella* sp. Raven (1976) has calculated the energy and reductants required for growth of phototrophically grown cells and showed that a minimum of 4.4 to 5.6 NADPH are required per C assimilated. He showed further that all the reductants and ATP required could be supplied from photoreactions via cyclic and non-cyclic photophosphorylation using H_2O as election donor. Thus *Chlorella* sp. and other photosynthetic plants possess an advantage over animals in having ample additional sources of energy and reducing potential. Consequently any partial blockage of the TCA cycle is less severe.

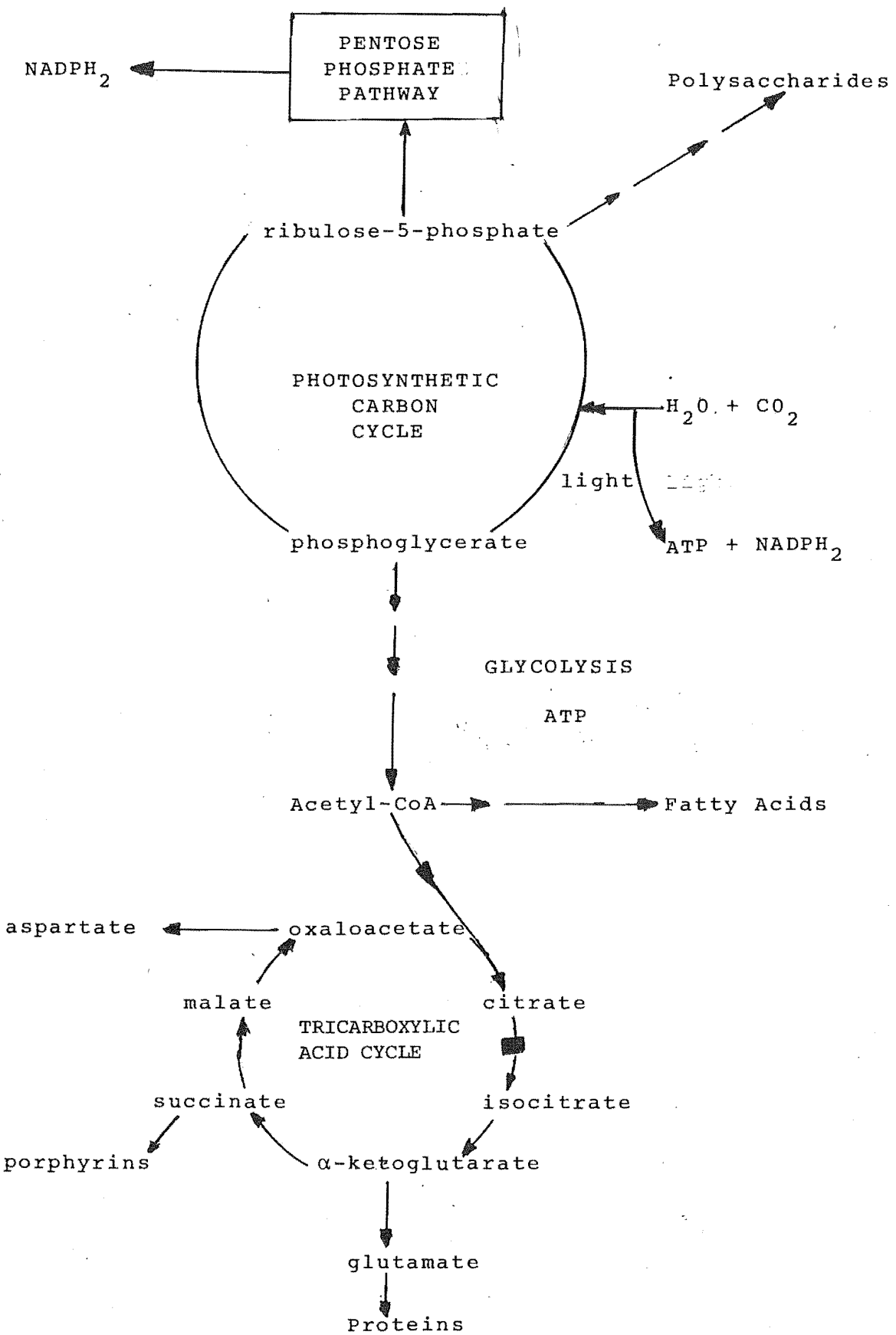
Nevertheless blockage of the TCA cycle could deprive cells of a source of intermediates for biosynthesis of proteins and carbohydrates. The intermediates of prime importance for amino acid synthesis, and subsequent protein synthesis are oxaloacetate and α-ketoglutarate. Reductive amination of these two intermediates forms the precursor amino acids, glutamate and aspartate, which via transamination with other keto-acids form the common amino acids of proteins. Glycine and serine are formed from photorespired glycolate. Oxaloacetate can be formed by dark CO_2-fixation other than via TCA cycle. Glutamate synthesis in *Chlamydomonas reinhardii* (Kates & Jones, 1964), and *Chlorella pyrenoidosa* (Hiller, 1964) has been shown to be inhibited by fluoroacetate but no decline in glutamine level was detected, indicating that in some algae, glutamate was not the only precursor of glutamine. Similarly, photosynthetic intermediates could provide the skeletons for synthesis of carbohydrates and fatty acids and these processes have been demonstrated to occur in chloroplasts. These various routes of biosynthesis are summarised in Figure 5.7.

In multicellular plants, as in the duckweeds, where there is increasing specialisation in the functions of cells, the transport and meristematic tissues are photosynthetically less active and the few chloroplasts present in these cells serve mainly for ATP and reductant generation. These cells suffer a further disadvantage over photosynthetic cells since the latter contain more substrates to compete with any fluorocitrate formed.

In addition, non-photosynthetic cells are far more dependent on their TCA cycle for the supply of energy and carbon skeletons.

Thus the role of photosynthesis and photophosphorylation cannot be disregarded in considering the relative tolerance of plants to fluorocitrate, a derivative of fluoroacetate and more specifically in those plants which synthesise fluoroacetate. It has frequently been reported that these plants are more toxic during their active growth period (Badenhuizen & Slinger, 1954; Vickery & Vickery, 1972), which implicates an association between tolerance to high fluoroacetate concentration and active growth made possible by active photosynthesis. Further support for such an association is the finding by Vickery & Vickery (1972) that fluoroacetate content was highest in young leaves (active) and

Fig: 5.7 Energy generating and biosynthetic pathways in a photosynthetic cell



lowest in roots (least active in terms of photosynthesis) in *D. toxicarium*. A similar distribution of fluoroacetate in various parts of the plant, *A georginae* was also reported by Oelrichs & McEwan (1962). The relatively higher fluoroacetate content of storage tissues such as seeds of *D. toxicarium* and *A. georginae* suggest that these tissues may act as sinks for the removal of toxic material from metabolically active non-photosynthetic tissues. Thus their findings suggest an association between the distribution of fluoroacetate content and the functional cell types in terms of metabolic, biosynthetic and photosynthetic activity. It therefore seems possible that tolerance of plants to NaFa may be conferred by their photosynthetic systems.

5.4.2. Effect of NaF on Duckweeds

NaF is toxic to duckweeds, but not as toxic as NaFa. For all the duckweeds studied the lethal concentrations were 20 mM NaFa as compared to 0.5-1.0 mM NaFa. Sublethal NaF concentrations resulted in smaller fronds and premature senscence developed. Fluoride injury to vegetation is well documented in the literature mainly with reference to pollution emitted from brickworks, or from factories producing aluminium and phosphate fertilisers, and plants growing in their vicinity suffer from severe foliar lesions and reduced growth (Thomas & Alther, 1966; Lovelace *et. al.* 1968; Miller *et. al.*, 1973). Similar observations were also reported on *Helianthus annuus* (Cooke, 1976c), *Pinus taeda* and *Acer rubrum* (Davis & Barnes, 1973) and other plants exposed to NaF.

The biochemical basis of F^- injury to plants has been a subject of intensive research. It is generally believed that senescence or chlorosis is a consqeuence of reduced metabolic activity (McCune & Weinstein, 1971). Inorganic fluoride (or F^-) is an inhibitor of many enzymes involved in respiratory pathways, one of the more important of which is enolase which catalyses the conversion of 2-phosphoglyceric acid to phsophoenolpyruvate (Thomas & Alther, 1966), an essential step in the glycolytic pathway. It also complexes with cytochrome C which is required for TCA cycle operation. Inhibition by F^- at these sites results in lowered metabolic activity, and may activate the pentose phosphate pathway. Studies on glucose metabolism in the leaves of *Chenopodium murale* and *Polygonium orientale* (cited by Thomas & Alther, 1966) indicated that respiration tended to utilise

more the pentose phosphate pathway in the presence of NaF. The continued operation of this pathway may explain the survival and growth of duckweeds at sublethal concentrations of NaF ($\leq$ 20 mM). The suppression of glycolysis and consequent reduction of TCA cycle activity in the presence of NaF could lead to reduced biosynthetic activity owing to shortage of ATP and carbon skeletons. It has been shown that the synthesis of chlorophyll in the leaves of fruit trees (apricot, cherry and apple) was reduced and its decomposition accelerated in the presence of 0.1-1.0 mM NaF; and that the carotenoid concentration in etiolated wheat sprouts decreased rapidly in the presence of 1.0 mM NaF. Breakdown of these pigments may explain bleaching of fronds of duckweeds grown in the presence of NaF.

Inorganic fluoride is also an inhibitor of three enzymes involved in the biosynthesis of cell wall polysaccharides viz: (Thomas & Alther, 1966):

(a) Uridine-diphosphoglucose dehydrogenase which catalyses the oxidation of UDP-glucose to UDP-glucuronic acid, a precursor of xylan and hemicellulose;

(b) Fructose-1,6 - diphosphatase which catalyses the conversion of fructose - 1,6 -diphosphate to fructose-6 - phosphate, an earlier intermediate; and

(c) phosphoglucomutase which catalyses the conversion of glucose-6 -phosphate to glucose-1-phosphate, an earlier precursor. The smaller fronds developed in the presence of NaF may be a manifestation of F^- inhibition at these sites of cell wall polysaccharide biosynthesis.

At equal concentrations of fluorine, NaFa-F was more toxic than NaF-F. The basis of the difference may lie at the relative importance of the sites of inhibition by NaFa or NaF. Although the sites of NaF inhibition may be multiple, these may not be the rate-limiting steps in the generation of ATP or carbon intermediates for growth whereas lethal synthesis of fluorocitrate from fluoroacetate and subsequent blockage of TCA cycle may be a rate-limiting step so that NaFa appeared to be more toxic than NaF. Therefore the environment of the area in which NaFa has been applied as a pesticide would be more toxic during the breakdown than after it is completely broken down by microbial activity.

The question, however, remains as to the fate of NaF or F^-

absorbed into the fronds. At toxic concentration the frond tissues deteriorated so that F^- may be subsequently released into the environment. At sublethal concentration, F^- absorbed may accumulate in the fronds to a concentration high enough to cause eventual death as a result of suppressed metabolic activity. It is also possible that complete suppression of metabolic activity could result from total inhibition of enzymes mentioned above. Alternatively blockage of the TCA cycle may eventuate via the synthesis of fluorocitrate and the latter possibility has been a subject of exploration for many workers in the field of fluoride metabolism in plants. Evidence exists in the literature that plants exposed to high concentration of F^- are able to assimilate it and produce fluoroacetate and fluorocitrate found in significant concentration: Cheng *et. al.* (1968) detected 6 µg fluoroacetate g^{-1} (dry weight) plant tissue and 140 µg fluorocitrate g^{-1} in *Glycine max* exposed to 43 ppb (parts per billion, USA) HF; Lovelace *et. al.*, (1968) reported the presence of 179 µg fluoroacetate and 896 µg fluorocitrate g^{-1} in *Medicago sativa* and *Agropyron cristatum* grown in the vicinity of a phosphate manufacturing plant; high concentrations of fluorocitrate were also detected in *A. georginae* (Preuss *et. al.*, 1970); and 200 mg fluoroacetate kg^{-1} was synthesised in *D. toxicarium* after 10 days of exposing the petioles to 1.0 mM NaF solution (Vickery & Vickery, 1975); and single cell cultures of *Glycine max*, *Thea sinensis* and *A. georginae* were reported to assimilate F^- and convert it into fluoroacetate and fluorocitrate (Peters & Shorthouse, 1972a,1972b). It thus appears that the ability of plants to assimilate F^- and incorporate it into toxic fluorocitrate is quite widespread. However evidence to the contrary also exists.

Cooke *et. al.*, (1976b) examined a wide range of plant species colonising fluorspar (CaF_2) mine waste and was unable to detect fluoro-organic compounds in these plants. Similarly fluoroacetate was not detected in tomato plants, *Glycine max*, corn, alfalfa, and crested wheat grass treated with NaF (Cited by McEwan, 1978) and fluoroacetate was not detected in 14-months old *A. georginae* seedlings planted in NH_4F-treated soils (Hall, 1974).

In view of the current evidence, F^- toxicity to duckweeds or plants in general, may involve not only multiple inhibition at various sites of metabolism but also the possibility of lethal synthesis of fluorocitrate.

5.4.3. Effect of the presence of both NaFa and NaF on growth of *S. oligorrhiza*.

In the presence of both NaFa and NaF, the reduction in growth rate constant of *S. oligorrhiza* was greater than when either compound was present alone. The reduction due to increase in NaFa concentration was greater than that due to increase in NaF concentration. This may be explained in terms of energetics, since the rate of growth depends on the availability of energy and carbon skeletons for which TCA cycle is the main pathway involved. The efficiency of the TCA cycle for ATP generation is 16 times that of glycolysis, so that blockage of the TCA cycle by fluorocitrate (assuming NaFa exerts its toxic effect on duckweeds via fluorocitrate) results in a substantial reduction in energy supply more critical than the consequences of a reduced rate of glycolysis. Consequently inhibition by NaFa ultimately limits the energy-producing steps and this masks the effect of NaF.

The environmental implication of this finding is that the enviornment would be most toxic during the breakdown of NaFa, less so when first applied and least after its complete breakdown.

CONCLUDING DISCUSSION

This project has been concerned mainly with studies on the biodegradability of sodium monofluoroacetate (NaFa) and its phytotoxicity. The biodegradation of NaFa was studied at soil, cellular and enzymatic levels. Soils treated with 2,500 ppm NaFa lost about 10% of the NaFa-F as soluble, free inorganic fluoride ions (F^-) whithin 14 weeks of incubation at 25°C. This suggests that the remaining NaFa-F may stay as NaFa; or decomposed with most of the F^- being complexed or adsorbed and rendered undetectable with the F^--specific electrode. The isolation of a variety of NaFa-degrading bacteria and fungi from these soils supports the deduction that much of the NaFa initially applied had been degraded. Furthermore the finding of a close parallel between increase in microbial numbers and F^- released suggests that the decomposition of NaFa is biological.

Pure culture studies using the fungi isolated from soils (*F. solani*, *F. oxysporum*, *A. strictum*, *Penicillium* sp. and *Aspergillus* sp.); and a bacterium, *Pseudomonas* sp. indicate further the close correspondence between biomass increase and NaFa defluorination under varying environmental conditions. The effect of such environmental factors as pH, temperature, aeration and nutrient status was investigated. pH determines the state of ionisation of fluoroacetate and therefore its availability to the organism. Whilst the growth of *F. solani* had an optimal pH plateau between 5.8 and 7.2, the defluorination of NaFa was more sensitive to pH with an optimum at 5.8. The difference in the response of growth and defluorination may be due to the effect of pH on the ionisation of fluoroacetate and other essential factors such as ammonium and phosphate ions. At higher pH (7.8) both growth and NaFa defluorination were significantly lower than the values observed at pH 5.8. At pH 7.8, a high percentage of fluoroacetate would be dissociated and its entry into the mycelium may depend on active transport. It is not known if an active transport mechanism of fluoroacetate was involved. Considering the relatively poor growth yield and the correspondingly lower percentage of NaFa degraded, the transport of fluoroacetate across the cell membrane is probably passive. Thus fluoroacetate would be made more available at lower pH's (undissociated fluoroacetate) which would favour entry by diffusion.

Both fungal growth and defluorinating activity were

enchanced by increasing the incubation temperature from 10°C to 37°C. At temperatures where growth was not evident, at or above 40°C, or below 10°C, F^- release was also undetectable. Increased aeration of the growth medium had no significant effect on both growth and NaFa defluorination by *Penicillium* sp. but slightly reduced the amount of NaFa degraded by *F. solani*.

Nutrient status plays an important role in influencing the ability of organisms to defluorinate NaFa. The effect of the types and concentrations of nitrogen or carbon sources on both the growth and defluorinating activity of the fungi was studied. It was found that while *F. solani* preferred NH_4^{-1} rather than NO_3^- or urea as a nitrogen source, such selectivity was not evident with *A. strictum* which showed equally poor growth and defluorinating activity in either NH_4^+ or NO_3^- media. The apparent difference in the relative activity (in terms of growth and NaFa defluorination) between fungal species in similar culture media or the same species in different culture media (as in the form of nitrogen avaialble) may be the result of some limiting factors such as vitamins and/or essential cofactors or metal ions required: for example the reduction of NO_3^- requires Cu^{++} and Mo^{6+}. Whilst growth and defluorinating activity could be enhanced by increasing nitrogen concentration, NH_4^+ was found to be toxic at high concentration.

The availability of another carbon source (glucose or acetate) enables such fungi as *Penicillum* sp. and *Aspergillus* sp. to grow and defluorinate NaFa. Unlike *F. solani*, *F. oxysporum* and *A. strictum*, *Penicillium* sp. and *Aspergillus* sp. were unable to grow and defluorinate NaFa in a medium with NaFa as the sole carbon source. Both growth and defluorinating activity of all fungi tested were enhanced in the presence of glucose or acetate. A small difference was found in the response of *Penicillium* sp. and *F. solani* to increasing glucose concentration: increase in growth and NaFa defluorination by *Penicillium* sp. was proportional to increasing glucose concentration whereas for *F. solani*, the degree of enhancement in NaFa defluorination decreased with increasing glucose concentration. This inverse relationship was also found for *F. solani* with acetate as the additional carbon source. With either glucose or acetate as the additional carbon source, this inverse relationship seen on

NaFa deflourination was not reflected in the growth yield which was approximately proportional. The difference in the response in growth and NaFa defluorination by *F. solani* suggests the possibility that substrate competition at the site of entry or at the metabolic level may be operating, probably with glycolate as the principle intermediate involved. It could therefore be concluded that, under environmental conditions which favour microbial growth, NaFa would be unlikely to persist.

The present work shows that the enzyme (fluorohydrolase) catalysing the breakdown of NaFa, was inductive and that the breakdown occurred inside the cell. Enzymatic studies showed that the breakdown products of NaFa were F^- and glycolate, the former was excreted and the latter assimilated in intact cells. The activity of fluorohydrolase was inhibited by F^- (probably via end-product inhibition), and -SH alkylators such as *p*-hydroxy-mercuribenzoate and heavy metals (Ag^+, Hg^{++}) suggesting the involvement of -Sh groups at the active site of the enzyme. Fluorohydrolase activity was unaffected in the presence of Mg^{++}, Fe^{++} or NaN_3, suggesting that the enzyme may have no metal ion requirements.

The optimal pH for fluorohydrolase (from either *F. solani* or *Pseudomonas*, sp.) activity was at pH 8.0, higher than that observed with intact mycelium (pH 5.8) or for the resting bacterial cells (pH 5.8-7.2) assayed at 25°C. This difference is in accord with the suggestion made earlier that the availability of fluoroacetate is determined by its state of ionisation, which is dependent on pH, and is the primary factor involved. In the presence of a membrane barrier, entry into intact cells by diffusion would be favoured at lower pH, whilst in its absence, the substrate is readily available. Both the bacterial and fungal fluorohydrolases were highly sensitive to pH below 8.0, suggesting that both enzymes may possess common ionisable groups on their polypeptide chains; or that only dissociated fluoroacetate is involved, or high OH^- concentration is required in the defluorination of NaFa.

Similar differences were also seen with respect to the temperature coefficients of the enzyme and cells. The temperature coefficients of NaFa defluorination by intact cells was about 4 times

higher than that by fluorohydrolase. This difference could be the result of diffusion-dependent availability of fluoroacetate to the intact cells.

Specificity studies show that the enzymes were highly specific for fluoroacetate, but relatively less active on fluoroacetamide, chloroacetate and bromoacetate. Iodoacetate, being an alkylator of -SH was a poor substrate for the enzymes. Tests on other fluoro-organic compounds show that fluorohydrolase was specific for the radical:

$$X - CH_2 - COZ \quad \text{where} \quad X = F, C, Br; \; Z = NH_2 \text{ or } OH$$

When considering the use of NaFa as a pesticide, there is a need to estimate the toxicity due to residual NaFa after application. Current practice used in the estimation of toxicity is based on total fluoride analysis after physical combustion of the baits (carrots, bran and molasses, meat etc.) to liberate fluoride ions from remaining NaFa. Results from this research project suggest the feasibility of using bacteria, fungi, or their fluorohydrolases, as a means of splitting the C-F bond of fluoroacetate under physiological conditions. This biological method could be more specific, rapid, simpler and cheaper in terms of equipment required, especially for routine analysis of residual NaFa in baits.

The biodegradability of NaFa does not rule out the possibility of its short-term adverse effect on other susceptible organisms such as nitrifying bacteria, photosynthetic bacteria and other beneficial soil flora or fauna. From the work on *Chlorella* sp. and the duckweeds: *S polyrrhiza, S. oligorrhiza* and *L. minor*, it was found that *Chlorella* sp. was tolerant to 20-mM NaFa whereas the duckweeds were killed in the presence of 0.5-1.0 mM NaFa. At sublethal concentrations of NaFa, reduced growth rate and premature senescence of the fronds of the duckweeds were observed. However, the sensitivity of the duckweeds to NaFa was population-dependent. While the high sensitivity of the duckweeds may suggest their usefulness as indicator organisms in waterways contaminated with NaFa, the population-dependent characteristic may limit their usefulness.

The short-term effect on other beneficial micro-organisms has not been investigated adequately. It has been shown from *in vitro* studies that nitrogenase activity of *Gloeocapsa* sp. was inhibited by fluoroacetate (Gallon *et. al.*, 1978). NaFa may interfere with such functional relationships as mycorrhiza.

In view of the finding that NaFa is readily biodegradable, it is unlikely that NaFa could pose a long-term hazardous effect and F^-, a product of NaFa breakdown is relatively less toxic than NaFa. Under the same test conditions for phytotoxicity 20 mM NaF was the lethal concentration for the duckweeds. Nevertheless, F^-, when present in sufficiently high concentration is toxic to both plants and animals. It is therefore, conceivable that NaFa and/or F^- may be assimilated and transformed into toxic fluoro-organic compounds by plants, animals and micro-organisms.

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TEDDYBEAR, PROGRAM BY J.B.WILSON, VERSION 1978 DEC 1, MANUAL 2.4
TITLE  EFFECT OF GLUCOSE AND FLUOROACETATE ON MYCELIAL GROWTH

+-----------------------------------------------------------+
|  EFFECT OF GLUCOSE AND FLUOROACETATE ON MYCELIAL GROWTH   |
+-----------------------------------------------------------+

NAMES  F 12468, G ABCDEF, R 1234

+-------------------------+
|  TREATMENT FACTORS :    |
|    F    1 2 4 6 8       |
|    G    A B C D E F     |
|  REPLICATION FACTORS :  |
|    R    1 2 3 4         |
+-------------------------+

COMMENT  TREATMENT F = FLUOROACETATE (1,2,4,6,8 )                    &
                   G = GLUCOSE (0.0,0.1,0.5,0.8,1.0,1.5 )

EXTRACT R

DATA  11.8 12.0 11.9 12.2  14.2 13.2 12.0 12.0  20.8 20.8 20.8 20.8    *    * &
    *    *  31.8 31.4 34.2 33.6  38.0 39.8 39.0 41.0  12.0 11.8 12.0 12.0      &
 10.2 10.9 10.8 12.8  19.4 19.4 19.4 19.4  27.5 26.0 29.0 27.5  32.8 29.8 30.1 &
 31.2  34.0 33.4 34.2 33.6   10.3 9.5 11.8 9.6  11.8 12.0 13.0 10.2  18.6 18.4 &
 18.4 18.6  21.0 21.5 21.4 24.6  28.4 28.0 29.0 30.0  31.4 32.8 29.8 32.8      &
 9.5 9.8 9.2 10.0  11.6 10.8 11.1 11.1  14.5 14.6 14.4 15.0  18.3 17.0 18.6    &
 16.6  27.4 27.0 26.6 28.2  27.1 28.2 26.0 28.2   8.6 7.6 9.0 7.9  10.7 10.0    &
 11.4 10.8  11.9 12.5 12.7 12.0  17.5 18.0 17.6 18.6  26.8 25.8 24.0 24.4  26.8&
 28.6 25.0 29.6

*****  THERE ARE TOO MANY MISSING VALUES  *****
       FOR TREATMENTS  F1   GE

*****  THERE ARE TOO MANY MISSING VALUES  *****
       FOR TREATMENTS  F1   GE

MISSING DATA ESTIMATION
=======================

THE ESTIMATION STOPPED AFTER 2 ITERATIONS, WHEN THE MAXIMUM RELATIVE CHANGE WAS  0.00461


TEDDYBEAR : EFFECT OF GLUCOSE AND FLUOROACETATE ON MYCELIAL GROWTH

DATA
====

 F1   GA        11.800        12.000        11.900        12.200
 F1   GB        14.200        13.200        12.000        12.000
 F1   GC        20.800        20.800        20.800        20.800
 F1   GD   *    20.775   *    20.650   *    20.705   *    21.070
 F1   GE        31.800        31.400        34.200        33.600
 F1   GF        38.000        39.800        39.000        41.000
 F2   GA        12.000        11.800        12.000        12.000
 F2   GB        10.200        10.900        10.800        12.800
 F2   GC        19.400        19.400        19.400        19.400
 F2   GD        27.500        26.000        29.000        27.500
 F2   GE        32.800        29.800        30.100        31.200
 F2   GF        34.000        33.400        34.200        33.600
 F4   GA        10.300         9.500        11.800         9.600
 F4   GB        11.800        12.000        13.000        10.200
 F4   GC        18.600        18.400        18.400        18.600
 F4   GD        21.000        21.500        21.400        24.600
 F4   GE        28.400        28.000        29.000        30.000
 F4   GF        31.400        32.800        29.800        32.800
 F6   GA         9.500         9.800         9.200        10.000
 F6   GB        11.600        10.800        11.100        11.100
 F6   GC        14.500        14.600        14.400        15.000
 F6   GD        18.300        17.000        18.600        16.600
 F6   GE        27.400        27.000        26.600        28.200
 F6   GF        27.100        28.200        26.000        28.200
 F8   GA         8.900         7.600         9.000         7.900
 F8   GB        10.700        10.000        11.400        10.800
 F8   GC        11.900        12.500        12.700        12.000
 F8   GD        17.500        18.000        17.600        18.600
 F8   GE        26.800        25.800        24.000        24.400
 F8   GF        26.800        28.600        25.000        29.600

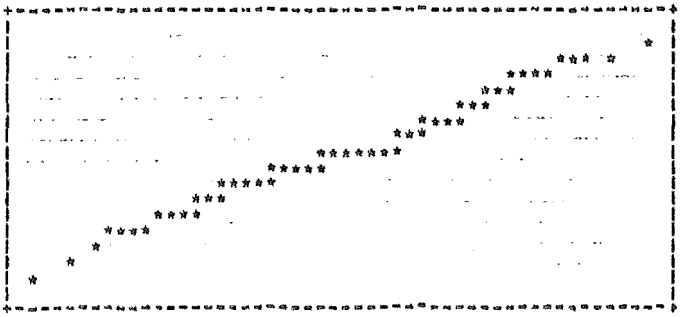
TESTS FOR NORMALITY ETC.
========================

IS THE DISTRIBUTION SKEWED TO ONE SIDE ?
     g1 =  0.10794, CRITICAL VALUE (P=0.05) = 0.4222,        N.S.

IS THE DISTRIBUTION THE WRONG SHAPE ? (2 KURTOSIS TESTS)
     g2 = -0.55459, CRITICAL VALUE (P=0.05) =-0.6985,        N.S.
     A  =  0.66207, CRITICAL VALUE (P=0.05) = 0.7389,        SIGNIFICANT

ARE THE ABSOLUTE VALUES OF THE RESIDUALS
     CORRELATED WITH THE FITTED (EXPECTED) VALUES ?
          R =  0.197663,  DF =   82,  P =  0.071499,        N.S.
     DITTO, USING THE RESIDUAL MEAN SQUARES AND THE MEANS
     FOR THE 30 COMBINATIONS OF TREATMENTS :
          R =  0.364985,  DF =   28,  P =  0.035659,        SIGNIFICANT

DISTRIBUTION PLOT :
     FOR A NORMAL
     DISTRIBUTION
     THIS SHOULD
     BE A
     STRAIGHT LINE
```



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GRAND MEAN = 20.210     COEFFICIENT OF VARIATION = 4.79%
```

TEDDYBEAR : EFFECT OF GLUCOSE AND FLUOROACETATE ON MYCELIAL GROWTH

ANALYSIS OF VARIANCE

	SS	DF	MS	F	P
F	696.990500	4	174.2476250	180.2294	0.000000
G	7949.624000	5	1589.9248000	1699.2958	0.000000
F G	353.703500	20	17.6851750	18.9013	0.000000
R	3.955082	3	1.3183606	1.4090	0.245967
ERROR	77.659861	83	0.9356610		
TOTAL	9082.132943	115	78.9750691		

FACTOR F

	MEAN	MS	SD	SE OF MEAN VARYING ERROR	SE OF MEAN CONSTANT ERROR
F1	23.164	0.98381907	0.99187654	0.20246595	0.19744841
2	22.467	0.75053455	0.86633397	0.17683968	0.19744841
4	20.538	1.32062670	1.14918523	0.23457645	0.19744841
6	17.950	0.45853906	0.67715512	0.13822371	0.19744841
8	16.992	1.17585033	1.08436909	0.22134591	0.19744841

DO THE 5 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 5.3390502, P = 0.254243, N.S.

DUNCAN'S NEW MULTIPLE RANGE TEST

F	8	6	4	2	1
P = 0.05					
P = 0.01					

FACTOR G

	MEAN	MS	SD	SE OF MEAN VARYING ERROR	SE OF MEAN CONSTANT ERROR
GA	10.425	0.40766122	0.63848353	0.14276926	0.21629389
B	11.530	0.93726421	0.96812407	0.21647912	0.21629389
C	17.120	0.08732630	0.29551024	0.06607810	0.21629389
D	21.195	1.42869620	1.19528080	0.26727291	0.21629389
E	29.025	1.22911126	1.10865290	0.24790233	0.21629389
F	31.965	1.65991638	1.28837742	0.28808995	0.21629389

DO THE 6 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 29.913544, P = 0.000015, SIGNIFICANT

DUNCAN'S NEW MULTIPLE RANGE TEST

G	A	B	C	D	E	F
P = 0.05						
P = 0.01						

TEDDYBEAR : EFFECT OF GLUCOSE AND FLUOROACETATE ON MYCELIAL GROWTH

FG MEANS

	A	B	C	D	E	F
F1	11.975	12.650	20.800	20.800	32.750	39.450
2	11.950	11.175	19.400	27.500	30.975	33.800
4	10.300	11.750	18.500	22.125	28.850	31.700
6	9.625	11.150	14.625	17.625	27.300	27.375
8	8.275	10.725	12.275	17.925	25.250	27.500

FACTOR R

	MEAN	MS	SD	SE OF MEAN VARYING ERROR	SE OF MEAN CONSTANT ERROR
R1	20.183	0.71447904	0.84526862	0.15432423	0.17660323
2	20.042	0.57975468	0.76141624	0.13901495	0.17660323
3	20.103	1.28415697	1.13320650	0.20689425	0.17660323
4	20.512	1.16425321	1.07900366	0.19699858	0.17660323

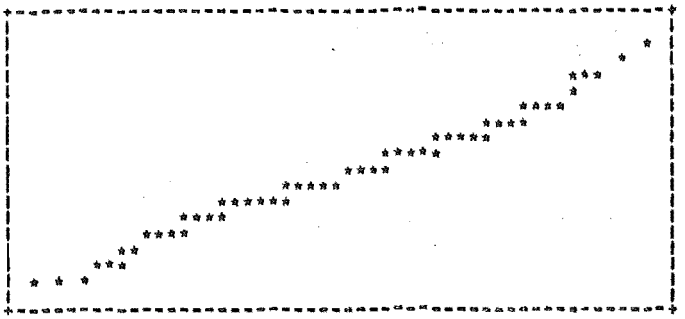
DO THE 4 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 4.3892968, P = 0.222380, N.S.

DUNCAN'S NEW MULTIPLE RANGE TEST

R	2	3	1	4
P = 0.05				

APPENDIX Ib

```
TEDDYBEAR, PROGRAM BY J.B.NILSON, VERSION 1978 DEC 1, MANUAL 2.4
TITLE  INTERACTION OF GLUCOSE AND FLUOROACETATE ON F DEGRADATION

+-----------------------------------------------------------+
| INTERACTION OF GLUCOSE AND FLUOROACETATE ON F DEGRADATION |
+-----------------------------------------------------------+

NAMES  F 12468, G ABCDEF,/R=10

+-------------------------+
| TREATMENT FACTORS :     |
|   F    1 2 4 6 8        |
|   G    A B C D E F      |
| REPLICATION FACTORS :   |
|   R    = 10             |
+-------------------------+

COMMENT  TREATMENT F = FLUOROACETATE (1,2,4,6,8 )                    &
                   G = GLUCOSE (0.0,0.1,0.5,0.8,1.0,1.5 )

EXTRACT R

TRANSFORM RAW DATA LN

DATA   4.0 3.0 4.8 3.5 3.8 4.0 4.8 3.8 4.0 3.8  8.0 5.6 5.6 5.0 5.8 7.4 6.4 5.8&
 4.6 7.8  8.0 7.8 7.2 8.0 7.4 7.8 6.6 7.4 8.2 7.6  11.0 11.0 9.2 11.0 9.6 10.0 &
 11.0 9.6 11.0 10.0  8.2 9.6 10.0 11.0 8.0 9.0 7.0 9.0 10.0 9.6  5.8 6.2 7.8  &
 9.4 8.0 8.8 8.0 6.6 7.6 6.2   5.2 3.5 6.0 5.2 3.3 3.8 4.4 5.4 3.9 5.2  4.8 5.1&
 7.2 4.3 5.6 5.0 4.6 5.6 5.4 6.2  11.0 13.0 12.0 13.0 14.0 14.0 14.0 13.0 13.0 &
 13.5  11.0 10.5 15.0 11.0 11.2 12.0 14.0 12.0 11.0 11.0  9.8 10.0 7.8 11.5  &
 12.0 9.4 10.0 10.0 14.0 10.5  9.2 8.6 10.0 9.6 10.0 10.0 9.4 11.0 10.0 9.0  &
 4.5 3.5 5.0 4.2 3.5 4.6 3.8 5.2 4.8 3.5  7.0 6.2 6.6 6.0 6.0 7.0 6.6 7.4 7.6  &
 5.6  15.0 17.0 18.0 15.0 13.0 13.0 17.0 13.0 16.0 17.0  14.0 16.0 16.0 14.5  &
 16.0 15.5 15.0 15.0 14.0 15.0  11.0 11.5 11.0 12.0 11.5 13.0 12.0 12.0 11.5  &
 11.5  14.0 16.0 16.0 15.0 15.0 14.0 13.0 13.0 13.0 15.5  4.6 4.8 4.2 5.6 4.6 &
 4.0 5.0 4.6 6.0 4.6  5.6 4.4 8.4 5.2 8.0 8.0 7.0 6.6 8.0 7.2  17.0 15.0 16.0  &
 17.0 17.0 17.5 17.0 16.0 18.0 16.0  13.5 13.5 14.0 13.5 15.0 17.0 14.0 14.5  &
 15.0 13.0  13.0 12.0 12.0 13.0 13.0 12.0 13.5 13.0 14.0 14.0  15.0 14.0 15.0 &
 17.0 14.0 14.5 16.0 16.0 14.5 15.5  5.6 5.4 5.8 5.4 5.2 5.0 5.4 5.2 5.4 8.0  &
 7.0 7.2 10.0 8.4 9.0 10.0 8.0 14.0 12.0 13.0  13.5 12.5 14.0 13.0 10.5 12.5  &
 13.0 12.0 10.0 12.0  16.0 16.0 16.0 15.0 16.0 16.0 16.0 16.0 18.0 17.0 14.0  &
 15.0 13.5 15.5 14.0 14.0 14.0 16.0 14.0 16.0  19.0 19.0 19.0 18.0 18.0 15.0  &
 16.0 20.0 15.0 20.0

TESTS FOR NORMALITY ETC.
========================

DO THE 30 COMBINATIONS OF TREATMENTS HAVE DIFFERENT ERROR VARIANCE ?
     BARTLETT'S TEST M/C = 81.220365,   P = 0.000001,        SIGNIFICANT

IS THE DISTRIBUTION SKEWED TO ONE SIDE ?
     G1 =  0.13906, CRITICAL VALUE (P=0.05) = 0.2455,        N.S.

IS THE DISTRIBUTION THE WRONG SHAPE ? (2 KURTOSIS TESTS)
     G2 =  0.57965, CRITICAL VALUE (P=0.05) = 0.5043,        SIGNIFICANT
     A  =  0.03669, CRITICAL VALUE (P=0.05) = 0.0218,        SIGNIFICANT

ARE THE ABSOLUTE VALUES OF THE RESIDUALS
     CORRELATED WITH THE FITTED (EXPECTED) VALUES ?
          R =  0.115790,  DF =  260,  P =  0.001266,         N.S.
     BIRTU, USING THE RESIDUAL MEAN SQUARES AND THE MEANS
     FOR THE 30 COMBINATIONS OF TREATMENTS :
          R =  0.303851,  DF =   28,  P =  0.102595,         N.S.


TEDDYBEAR : INTERACTION OF GLUCOSE AND FLUOROACETATE ON F DEGRADATION : RAW DAT

DISTRIBUTION PLOT:
     FOR A NORMAL
     DISTRIBUTION
     THIS SHOULD
     BE A
     STRAIGHT LINE
```



```
GRAND MEAN = 10.492     COEFFICIENT OF VARIATION = 10.72%

ANALYSIS OF VARIANCE
====================

                SS          DF          MS          F          P

F         1105.584867        4     276.3962167    218.3121   0.000000
G         3495.409367        5     699.0818733    552.1712   0.000000
F G        667.001133       20      33.3500567     26.3416   0.000000

R           16.275367        9       1.8083741      1.4283   0.175903

ERROR      330.441633      261       1.2660599
TOTAL     5614.712366      299      18.7783022

FACTOR F
========

          MEAN          MS          SD      SE OF MEAN      SE OF MEAN
                                          VARYING ERROR   CONSTANT ERROR

 F1       7.455   0.88119042   0.93871743     0.12118790     0.14526183
  2       9.187   1.24786731   1.11707981     0.14421438     0.14526183
  4      11.252   1.19409719   1.09274754     0.14107310     0.14526183
  6      11.785   0.95049566   0.97493307     0.12586340     0.14526183
  8      12.783   2.05664891   1.43410213     0.18514179     0.14526183

DO THE 5 LEVELS HAVE DIFFERENY ERROR VARIANCE ?
     BARTLETT'S TEST M/C = 12.224615,   P = 0.015757,        SIGNIFICANT

DUNCAN'S NEW MULTIPLE RANGE TEST

          F     1     2     4     6     8

P = 0.05

P = 0.01
```

TEDDYBEAR : INTERACTION OF GLUCOSE AND FLUOROACETATE ON F DEGRADATION : RAW DATA

FACTOR G

	MEAN	MS	SD	SE OF MEAN VARYING ERROR	SE OF MEAN CONSTANT ERROR
GA	4.652	0.47149489	0.68665466	0.09710766	0.15912636
B	6.926	1.87499527	1.36930467	0.19364892	0.15912636
C	13.060	1.46488646	1.21032494	0.17116579	0.15912636
D	13.562	1.11212324	1.05457254	0.14913908	0.15912636
E	11.790	1.14789329	1.07139642	0.15151834	0.15912636
F	12.964	1.52496922	1.23489644	0.17464073	0.15912636

DO THE 6 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 21.403823, P = 0.000679, SIGNIFICANT

DUNCAN'S NEW MULTIPLE RANGE TEST

	G	A	B	E	F	C	D
P = 0.05					-------		
P = 0.01					----------		

FG MEANS

	A	B	C	D	E	F
F1	3.950	6.200	7.600	10.340	9.200	7.440
2	4.590	5.430	13.050	11.870	10.500	9.680
4	4.260	6.600	15.400	15.100	11.700	14.450
6	4.820	6.840	16.650	14.300	12.950	15.150
8	5.640	9.860	12.300	16.200	14.600	18.100

FACTOR R

	MEAN	MS	SD	SE OF MEAN VARYING ERROR	SE OF MEAN CONSTANT ERROR
R1	10.177	1.01425045	1.00710002	0.18387047	0.20543125
2	10.097	1.13435773	1.06506231	0.19445288	0.20543125
3	10.770	1.71158378	1.30827512	0.23885726	0.20543125
4	10.550	0.98879451	0.99438147	0.18154839	0.20543125
5	10.267	0.84894777	0.92138361	0.16822086	0.20543125
6	10.460	1.39539987	1.18127045	0.21566949	0.20543125
7	10.503	0.85240115	0.92325573	0.16856266	0.20543125
8	10.623	1.43718276	1.19882557	0.21887460	0.20543125
9	10.650	1.97638072	1.40583808	0.25666974	0.20543125
10	10.827	1.30130026	1.14074548	0.20827068	0.20543125

DO THE 10 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 9.6320548, P = 0.381085, N.S.

DUNCAN'S NEW MULTIPLE RANGE TEST

	R	2	1	5	6	7	4	8	9	3	10
P = 0.05		----------									

P = 0.01		----------									

```
TEDDYBEAR, PROGRAM BY J.B.NILSON, VERSION 1976 DEC 1, MANUAL 2.4
TITLE INTERACTION OF FLUORIDE AND FLUOROACETATE ON S. OLIGORRHIZA
```

APPENDIX IIa

```
+-----------------------------------------------------+
| INTERACTION OF FLUORIDE AND FLUOROACETATE ON S. OLIGORRHIZA |
+-----------------------------------------------------+

NAMES F 01234, A 01234,/R 12345

+-----------------------+
| TREATMENT FACTORS :   |
|   F    0 1 2 3 4      |
|   A    0 1 2 3 4      |
| REPLICATION FACTORS : |
|   R    1 2 3 4 5      |
+-----------------------+

COMMENT THE TREATMENTS ARE                                              &
        F=FLUORIDE F0=00 F4=0005                                        &
        A=FLUOROACETATE  A0=00 A4=0001

EXTRACT R

DATA 0.08d9107 0.0875238 0.093609 0.07971 0.082996 0.049651 0.000557 0.0678   &
0.064150 0.045019 0.045174 0.05142 0.03461 0.06339 0.06422 0.051598 0.020058  &
0.04465 0.025968 0.041089 0.03926 0.02553 0.03747 0.03761 0.03705 0.083547    &
0.063048 0.062949 0.0761303 0.0799569 0.05072 0.05576 0.02856 0.06892 0.05818 &
0.05532 0.04627 0.05591 0.04560 0.04134 0.02109 0.02207 0.03573 0.04677       &
0.03453 0.03195 0.03038 0.00803 0.01968 0.02048 0.06945 0.07770 0.07611       &
0.05145 0.072132 0.05366 0.05291 0.05469 0.04306 0.03972 0.04914 0.04113      &
0.05135 0.05009 0.03966 0.02728 0.02222 0.023006 0.025438 0.04259 0.026148    &
0.019099 0.02510 0.02395 0.01710 0.068658 0.073211 0.0750513 0.07252 0.06985  &
0.04553 0.04590 0.04641 0.03804 0.04059 0.03408 0.03841 0.04123 0.04134       &
0.02691 0.01325 0.01876 0.02222 0.01248 0.02052 0.02121 0.02535 0.01245       &
0.02260 0.035144 0.052403 0.056273 0.03469 0.050098 0.045625 0.04037 0.03158 &
0.03309 0.04447 0.03852 0.02947 0.03309 0.03269 0.03157 0.03159 0.01841       &
0.01407 0.01508 0.01891 0.01771 0.01754 0.01759 0.00941 0.00709 0.01547
```

TESTS FOR NORMALITY ETC.

DO THE 25 COMBINATIONS OF TREATMENTS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 42.710291, P = 0.010732, SIGNIFICANT

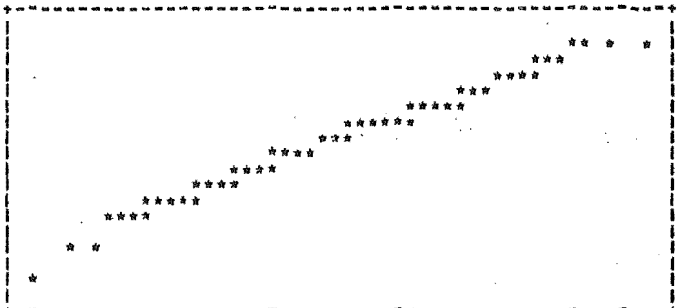
IS THE DISTRIBUTION SKEWED TO ONE SIDE ?
g1 = -0.25641, CRITICAL VALUE (P=0.05) =-0.3949, N.S.

IS THE DISTRIBUTION THE WRONG SHAPE ? (2 KURTOSIS TESTS)
g2 = -0.34899, CRITICAL VALUE (P=0.05) =-0.6603, N.S.
A = 0.1207, CRITICAL VALUE (P=0.05) = 0.0361, N.S.

ARE THE ABSOLUTE VALUES OF THE RESIDUALS
CORRELATED WITH THE FITTED (EXPECTED) VALUES ?
R = -0.003846, DF = 95, P = 0.970173, N.S.
DITTO, USING THE RESIDUAL MEAN SQUARES AND THE MEANS
FOR THE 25 COMBINATIONS OF TREATMENTS :
R = -0.000153, DF = 23, P = 0.999422, N.S.

TEDDYBEAR : INTERACTION OF FLUORIDE AND FLUOROACETATE ON S. OLIGORRHIZA

DISTRIBUTION PLOT :



GRAND MEAN = 0.042564 COEFFICIENT OF VARIATION = 17.88%

ANALYSIS OF VARIANCE

	SS	DF	MS	F	P
F	.00841474602	4	.00210368651	36.3051***	0.000000
A	.03912966425	4	.00978241606	168.8234***	0.000000
F A	.00152972703	16	.00009560798	1.6500 n.s.	0.070461
R	.00011006547	4	.00002751637	0.4749 n.s.	0.754071
ERROR	.00556268686	96	.00005794465		
TOTAL	.05474689024	124	.00044150718		

FACTOR F

	MEAN	MS	SD	SE OF MEAN VARYING ERROR	SE OF MEAN CONSTANT ERROR
F0	0.053753	0.00009698	0.00984773	0.00196955	0.00152243
1	0.047477	0.00009290	0.00963850	0.00192770	0.00152243
2	0.043527	0.00004540	0.00673761	0.00134752	0.00152243
3	0.038541	0.00003184	0.00564275	0.00112855	0.00152243
4	0.029520	0.00002261	0.00475468	0.00095098	0.00152243

DO THE 5 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 14.829556, P = 0.005068, SIGNIFICANT

DUNCAN'S NEW MULTIPLE RANGE TEST

F	4	3	2	1	0
P = 0.05			-------		
P = 0.01		-------			

TEDDYBEAR : INTERACTION OF FLUORIDE AND FLUOROACETATE ON S. OLIGORRHIZA

FACTOR A

	MEAN	MS	SD	SE OF MEAN: VARYING ERROR	SE OF MEAN CONSTANT ERROR
A0	0.072624	0.00002944	0.00542576	0.00108515	0.00152243
1	0.048113	0.00007713	0.00878235	0.00175647	0.00152243
2	0.042359	0.00005562	0.00745800	0.00149160	0.00152243
3	0.026253	0.00006059	0.00897096	0.00179539	0.00152243
4	0.023468	0.00004695	0.00685181	0.00137036	0.00152243

DO THE 5 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 5.8191157, P = 0.213070, N.S.

DUNCAN'S NEW MULTIPLE RANGE TEST

	A	4	3	2	1	0
P = 0.05		-------				
P = 0.01		-------				

FA MEANS

	0	1	2	3	4
F0	0.086550	0.058352	0.051763	0.036717	0.035384
1	0.081525	0.052432	0.049268	0.032038	0.022104
2	0.075368	0.048812	0.042270	0.026107	0.023079
3	0.071858	0.043254	0.036794	0.017446	0.023351
4	0.047818	0.037726	0.031682	0.016956	0.013420

FACTOR B

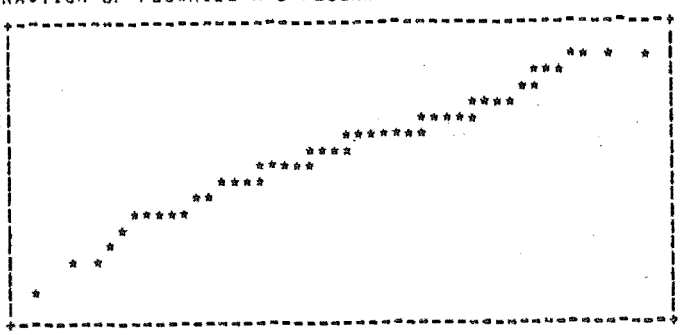
	MEAN	MS	SD	SE OF MEAN: VARYING ERROR	SE OF MEAN CONSTANT ERROR
R1	0.043345	0.00004260	0.00652709	0.00130542	0.00152243
2	0.042100	0.00004075	0.00638395	0.00127679	0.00152243
3	0.041069	0.00009801	0.00989969	0.00197998	0.00152243
4	0.043722	0.00005881	0.00766891	0.00153378	0.00152243
5	0.042564	0.00004955	0.00703868	0.00140778	0.00152243

DO THE 5 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
BARTLETT'S TEST M/C = 5.1294340, P = 0.274273, N.S.

DUNCAN'S NEW MULTIPLE RANGE TEST

	R	3	2	5	1	4
P = 0.05		----------				

```
TEDDYBEAR, PROGRAM BY J.B.WILSON, VERSION 1978 DEC 1, MANUAL 2.4
TITLE INTERACTION OF FLUORIDE AND FLUOROACETATE ON S. OLIGORRHIZA

+-----------------------------------------------------------+
|  INTERACTION OF FLUORIDE AND FLUOROACETATE ON S. OLIGORRHIZA |
+-----------------------------------------------------------+

NAMES  T ABCDEFGHIJKLMNOPQRSUVWXYZ,/R 12345

+-----------------------------------------------------------+
|  TREATMENT FACTORS :                                      |
|    T    A B C D E F G H I J K L M N O P Q R S U V W X Y Z |
|  REPLICATION FACTORS :                                    |
|    R    1 2 3 4 5                                         |
+-----------------------------------------------------------+

COMMENT  A = F0A0, B = F0A1, C = F0A2, D = F0A3, E = F0A4                    &
         F = F1A0, G = F1A1, H = F1A2, I = F1A3, J = F1A4,                   &
         K = F2A0, L = F2A1, M = F2A2, N = F2A3, O = F2A4,                   &
         P = F3A0, Q = F3A1, R = F3A2, S = F3A3, U = F3A4,                   &
         V = F4A0, W = F4A1, X = F4A2, Y = F4A3, Z = F4A4.

DATA 0.0889107 0.0875238 0.093609 0.07971 0.082990 0.049651 0.060557 0.0678    &
0.064150 0.0466019 0.045174 0.05142 0.03461 0.06339 0.06422 0.051598 0.020058  &
0.04485 0.025980 0.041089 0.03926 0.02553 0.03747 0.03761 0.03705 0.083547     &
0.083044 0.082949 0.0781303 0.0799569 0.05072 0.05576 0.02856 0.06892 0.05818  &
0.05532 0.04827 0.05591 0.04560 0.04134 0.02109 0.02207 0.03573 0.04677        &
0.03453 0.03145 0.03038 0.00803 0.01968 0.02048 0.06945 0.07770 0.07611        &
0.08145 0.072132 0.05368 0.05291 0.05469 0.04306 0.03972 0.04912 0.04113       &
0.03135 0.05009 0.03966 0.02728 0.02222 0.023006 0.025438 0.04259 0.026148     &
0.019099 0.02910 0.02395 0.01710 0.060858 0.073211 0.0750513 0.07252 0.06985  &
0.04533 0.04590 0.04641 0.03804 0.04059 0.03408 0.03841 0.04123 0.04134        &
0.02891 0.01325 0.01876 0.02222 0.01248 0.02052 0.02121 0.02535 0.01245        &
0.02260 0.035144 0.052403 0.056273 0.03469 0.050098 0.045625 0.04037 0.03158  &
0.03369 0.04447 0.03452 0.02947 0.03309 0.03269 0.03157 0.03159 0.01841        &
0.01467 0.01508 0.01891 0.01771 0.01754 0.01759 0.00941 0.00709 0.01547

TESTS FOR NORMALITY ETC.
========================

IS THE DISTRIBUTION SKEWED TO ONE SIDE ?
     G1 = -0.34034, CRITICAL VALUE (P=0.05) =-0.3875,        N.S.

IS THE DISTRIBUTION THE WRONG SHAPE ? (2 KURTOSIS TESTS)
     G2 =  0.15695, CRITICAL VALUE (P=0.05) = 0.7717,        N.S.
     A  =  0.02942, CRITICAL VALUE (P=0.05) = 0.0354,        N.S.

ARE THE ABSOLUTE VALUES OF THE RESIDUALS
     CORRELATED WITH THE FITTED (EXPECTED) VALUES ?
          R = -0.018308, DF =  99, P = 0.855806,             N.S.
     DITTO, USING THE RESIDUAL MEAN SQUARES AND THE MEANS
     FOR THE 25 COMBINATIONS OF TREATMENTS :
          R = -0.022625, DF =  23, P = 0.914514,             N.S.


TEDDYBEAR : INTERACTION OF FLUORIDE AND FLUOROACETATE ON S. OLIGORRHIZA
DISTRIBUTION PLOT :

     FOR A NORMAL
     DISTRIBUTION
     THIS SHOULD
     BE A
     STRAIGHT LINE
```



```
GRAND MEAN = 0.042564     COEFFICIENT OF VARIATION = 17.70%

ANALYSIS OF VARIANCE
====================

                SS          DF          MS          F          P
T          .04907413791      24    .00204475575    36.0452    0.000000
ERROR      .00567275233     100    .00005672752
TOTAL      .05474689024     124    .00044150718

FACTOR T
========
          MEAN          MS          SD      SE OF MEAN     SE OF MEAN
                                            VARYING ERROR  CONSTANT ERROR
 TA    0.086550    0.00002894    0.00537976    0.00240590    0.00336831
  B    0.058352    0.00007000    0.00836683    0.00374176    0.00336831
  C    0.051763    0.00015703    0.01253118    0.00560411    0.00336831
  D    0.036717    0.00017483    0.01322250    0.00591328    0.00336831
  E    0.035384    0.00003105    0.00557240    0.00249205    0.00336831
  F    0.081525    0.00000560    0.00236678    0.00105846    0.00336831
  G    0.052432    0.00022195    0.01489814    0.00666265    0.00336831
  H    0.049266    0.00003951    0.00628578    0.00281109    0.00336831
  I    0.032038    0.00011402    0.01067816    0.00477542    0.00336831
  J    0.022164    0.00009301    0.00964399    0.00431293    0.00336831
  K    0.075368    0.00002212    0.00470303    0.00210326    0.00336831
  L    0.048012    0.00004770    0.00690637    0.00308862    0.00336831
  M    0.042270    0.00005886    0.00767191    0.00343098    0.00336831
  N    0.026107    0.00006956    0.00834029    0.00372989    0.00336831
  O    0.023079    0.00002450    0.00495022    0.00221381    0.00336831
  P    0.071656    0.00000668    0.00258544    0.00115625    0.00336831
  Q    0.043254    0.00001389    0.00372673    0.00166665    0.00336831
  R    0.036794    0.00002812    0.00530260    0.00237149    0.00336831
  S    0.017446    0.00001906    0.00436562    0.00195237    0.00336831
  U    0.023351    0.00006676    0.00817084    0.00365411    0.00336831
  V    0.047018    0.00003872    0.00828947    0.00370716    0.00336831
  W    0.037726    0.00002679    0.00517603    0.00231479    0.00336831
  X    0.031682    0.00000198    0.00140646    0.00062899    0.00336831
  Y    0.016956    0.00000361    0.00195230    0.00087310    0.00336831
  Z    0.013420    0.00002368    0.00486608    0.00217618    0.00336831

DO THE 25 LEVELS HAVE DIFFERENT ERROR VARIANCE ?
     BARTLETT'S TEST M/C = 49.396020,   P = 0.001689,        SIGNIFICANT
```

TEDDYBEAR I INTERACTION OF FLUORIDE AND FLUOROACETATE ON S. OLIGORRHIZA

DUNCAN'S NEW MULTIPLE RANGE TEST

	T	Z	Y	S	J	O	U	N	X	I	E	D	R	W	M	Q	V	L	H	C	G	B	P	K	F	A
P = 0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
								-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
									-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
										-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
												-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
														-	-	-	-	-	-	-	-	-	-	-	-	-
																	-	-	-	-	-	-	-	-	-	-
																									-	-
P = 0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
								-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
									-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
										-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
												-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
														-	-	-	-	-	-	-	-	-	-	-	-	-
																	-	-	-	-	-	-	-	-	-	-
																									-	-